PLATINUM CHEMOTHERAPY:

INVESTIGATION OF CELLULAR RESISTANCE MECHANISMS AND EARLY DEVELOPMENT OF AN ORAL ANALOGUE.

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ABSTRACT

The discovery and therapeutic development of the anticancer drug cisplatin was an important milestone in the treatment of people suffering from many tumours, especially teratoma and ovarian carcinoma. The main problem with the drug is its toxicity which necessitated the development of similarly active but better tolerated analogues, for example carboplatin. Despite demonstrating a more acceptable toxicity profile, carboplatin and other newer platinum chemotherapies are not ideal drugs both because of their side-effects and also because tumours may demonstrate primary or acquired platinum resistance.

In order to understand better the mechanisms by which tumours become resistant to platinum chemotherapy, tumour models have been developed for in vitro experiments. Some of these tumours are murine, but there is an increasing library of human tumour types which allow measurement of various cellular activities and enzymes believed to be relevant in drug resistance.

In this thesis, the results are presented of experiments performed to investigate the mechanisms of platinum resistance in the murine L1210 leukaemia cell line *in vitro*. In addition, platinum resistant L1210 variants have been developed and these cell lines are also studied.

In addition to allowing characterisation of the resistant cells, murine and human tumour models are invaluable in screening novel compounds for antitumour activity. In the second part of the thesis, the L1210 sensitive and resistant cell lines are employed along with human ovarian cancer cell lines *in vitro* to screen several compounds which are structurally related to the parent platinum drugs cisplatin, carboplatin, tetraplatin and iproplatin. The aim of the work was to identify compounds which demonstrated cytotoxic activity against cell lines which were known to be platinum resistant. The mixed amine platinum IV dichlorodicarboxylates were selected and data on their *in vitro* activities are presented. The six leading novel compounds were also tested for relative activity against the plasmacytoma tumour ADJ/PC6 in murine xenografts, and their relative activities and toxicities when delivered by the oral or the intraperitoneal route were investigated.

Finally, the pharmacokinetic profiles of the six lead compounds were studied in non-tumour bearing mice following oral delivery. The haematological toxicities are also presented from experiments in the murine model.

On completion of the initial studies detailed in this thesis, further experiments have resulted in selection of one of the six compounds, JM216, as a suitable candidate for evaluation in the clinic as an oral platinum antitumour agent.

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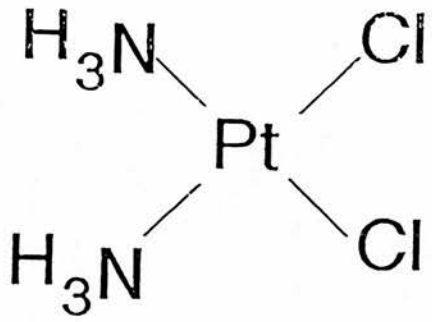
CHAPTER I

INTRODUCTION

1.1 Cisplatin: Discovery and mode of action.

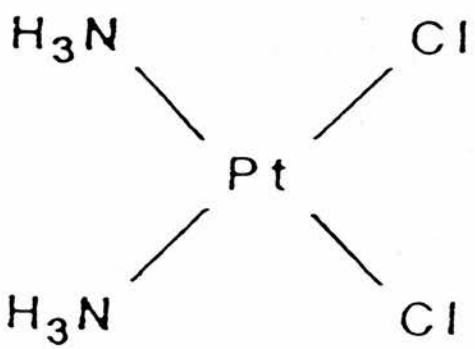
In 1965 Rosenberg reported in "Nature" [Rosenberg et al, 1965] that during investigation of the effects of an electric field on the growth processes in bacteria, his team discovered that within 1-2 hours of passing an electric current of 2 amp between platinum electrodes, E. coli bacteria ceased dividing and began to elongate. Within a few hours, all of the bacteria were in the form of elongated filaments with the increase in length continuing for two hours after removal of the voltage. Further study of the cause of the filamentous growth (defined as inhibition of cell division but not of growth) revealed that a platinum salt was the active agent, since inoculating the bacterial continuous culture chamber with a solution of $(NH_4)_2PtCl_6$ so as to maintain a concentration in the chamber of Pt(IV) 10ppm over 2 hours caused filaments to appear. More detailed investigation into the properties of one of the platinum-derived species revealed that the compound cis-platinum II diamminodichloride (cisplatin - structure shown in Figure 1.1) had significant growth inhibition effects on several transplantable rodent tumours, including the L1210 murine leukaemia, sarcoma 180 [Rosenberg and von Camp, 1970] Dunning ascitic leukaemia and Walker 256 carcinosarcoma [Kociba et al, 1970]. It was, therefore, only a matter of time until cisplatin was brought to clinical trials as a prospective antineoplastic drug.

FIGURE 1.1 STRUCTURE OF CISPLATIN

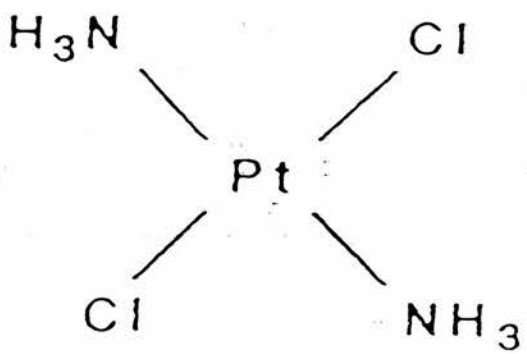


Cisplatin

FIGURE 1.2 CIS AND TRANS-PLATINUM STRUCTURES



cis-DDP



trans-DDP

Prior to its introduction to the clinic in 1971, the mechanism of action of cisplatin was poorly understood. Subsequently it has been established that in aqueous solution, both chlorine atoms are slowly displaced with water leading to the formation of a positively charged aquated complex [Leroy et al, 1979]. The activated complex then interacts with nucleophilic sites on DNA purine and pyrimidine bases to produce bifunctional covalent links [Zwelling and Kohn, 1979]. The end result is very similar to the effect produced by the alkylating antitumour drugs chlorambucil and melphalan. Further analysis identified the specific sites of action to be the N_7 of guanine or the N_3 of cytosine residues. The major adducts are intrastrand cross links formed by the binding of cisplatin to two neighbouring guanines or, less frequently, to adenine and guanine or to two guanines separated by one or more nucleobases [Roberts and Thomson, 1979; Zwelling and Kohn, 1979]. Interstrand cross-links between two guanines on complementary DNA strands, and DNA protein cross-links are also formed. Experiments were carried out to determine the importance of the position of the ammine and the chloride groups. The conclusion was that cis- compounds (Figure 1.2) were more effective than trans- compounds at inducing interstrand cross-links in DNA and could more readily induce links between adjacent guanines on the same strand of DNA [Roberts and Friedlos, 1987]. It has been established that the interstrand cross links account for less than 1% of all cross links, and this suggests that the intrastrand links are the more significant of the two [Eastman, 1983; Pascoe and Roberts, 1974].

Studies on the excision repair of platinum adducts on the DNA of various cell types confirmed that DNA is the target for the cytotoxic action of cisplatin. Cells which were exposed to cisplatin while in stationary phase and immediately diluted into fresh complete medium to release them from the stationary phase were more sensitive to cisplatin than were cells treated while in the exponential phase of growth. A reduction in excision repair capacity was the reason for the increased sensitivity, as demonstrated by the finding that excision-proficient exponentially growing cells removed more DNA-bound platinum prior to replication of their DNA than did stationary cells [Fraval and Roberts 1979]. This strongly supports the contention that it is the platinum bound to the DNA at initiation of DNA replication which determines cytotoxicity. Further, repair defective xeroderma pigmentosum cells are significantly more sensitive to cisplatin than are other cell lines.

The changes in DNA conformation induced by cisplatin result in inhibition of DNA synthesis thereby inducing a delay in the cells' progression through the S (DNA synthesis) phase. The toxic effect of cisplatin is therefore maximal when cells are treated at the intermitotic or G1 phase of the cell cycle [Fraval and Roberts, 1979].

It has been suggested that cisplatin can increase the naturally occurring cell-mediated lysis of tumour cells through increasing the tumour cell surface-associated antigens which permit the immunological recognition and

subsequent rejection of cisplatin-treated cells. The phenomenon was demonstrated in murine and in human tumour cells [Collins and Kao, 1989] and since it was shown that the lysis was performed by naturally occurring cytotoxic cells, the postulate is that these cells may provide a host defence mechanism which contributes to the anticancer potential of cisplatin.

1.1a Clinical activity.

Phase I trials with cisplatin were encouraging in that responses were seen in a variety of tumours including anaplastic thyroid carcinoma (1 patient), transitional cell carcinoma of the bladder (1 patient), breast cancer (1 patient) and testicular tumours (9 of 11 patients). The fact that three of the responses were complete (ie. no measurable tumour was seen after treatment) was the most exciting finding, and raised hopes that the platinum based drug might have a specific effect on tumours arising from the genitourinary tract [Higby et al, 1974].

Further trials with cisplatin confirmed its impressive efficacy in testicular tumours [Higby et al, 1973] and advanced ovarian cancer [Wiltshaw and Kroner, 1976]. Cisplatin was shown to have low single-agent activity in patients with lung cancer with only one response in an adenocarcinoma from a trial population of 17 patients when 75mg/m^2 cisplatin was given 3 weekly [Rossof et al, 1976]. Although cisplatin has a single agent activity of 30% in advanced urothelial tumours [Yagoda, 1983], it is now used

mainly in combination with other antineoplastic drugs - for example the curative combination of bleomycin, etoposide and cisplatin (BEP) in testicular tumours [Peckham, 1983].

Alternative routes of delivery of cisplatin have been explored in an attempt to ensure maximum concentration of drug at the site of the tumour without associated toxicity to normal surrounding tissues. Intraperitoneal administration of cisplatin as treatment for malignant mesothelioma was found to produce responses in 9 of 13 patients who had primary peritoneal disease. Of 8 patients treated for primary pleural disease, only 1 responded. Cisplatin was given at weekly doses of 90-100mg/m^2 with simultaneous intravenous administration of sodium thiosulphate as renal protection (see 1.1d) and was no more toxic than delivery by the intravenous route [Markman et al, 1986]. In a pilot study treating gastric cancer, adjuvant intraperitoneal cisplatin 60mg/m^2 was given to 18 patients. Radioisotope tracer studies in 6 patients confirmed good drug distribution throughout the peritoneal cavity. There was no WHO Grade III/IV toxicity and the median survival was 17 months [Jones et al, 1994]. Cisplatin has also been administered by intra-arterial injection in primary hepatocellular carcinoma with responses seen in 4 of 10 patients using relatively low doses of 50mg/m^2 [Kajanti et al, 1986]. In head and neck cancer, intra-arterial administration of cisplatin 10mg twice daily x5-10 days produced good response rates of 68%, but this was to be expected in a treatment-naiive

group of patients where the majority of tumours were squamous cell carcinomas [Frustaci et al, 1986]. The duration of the responses was not stated in the paper. Hyperthermic isolated limb perfusion with cisplatin in patients with advanced malignant melanoma of one leg resulted in complete responses in 12 of 15 patients in one study where the dose used was 75mg/m^2 [Klein and Ben-Ari, 1987]. At a mean follow-up of 36 months, 85% of the patients were alive and disease-free. Cisplatin has even been given by subconjunctival injection in rabbits which had been implanted with ocular melanoma in an attempt to penetrate this pharmacological sanctuary site [Skov et al, 1987]. A report by Deurloo and colleagues commented on the advantages in delivering cisplatin by a slow-release device [Deurloo et al, 1991]. Mice were exposed to drug given either by the intraperitoneal route or by starch or polymeric hydrogels. The slower release formulations achieved higher total tumour drug concentrations, but there was no homogeneous distribution of drug within the tumour.

1.1b High dose cisplatin.

In addition to experimenting with compartmental chemotherapy, efforts have been made to increase the response rate of cisplatin by dose escalation from standard 20mg/m^2 daily for 5 days to 40mg/m^2 daily for 5 days. There were responses in 3 of 6 patients with ovarian cancer whose tumours were resistant to the conventional dose schedule and with combination therapy for high risk non-seminomatous testicular carcinoma there was an 88%

complete response rate [Ozols et al, 1984]. In advanced head and neck cancer, high dose cisplatin therapy resulted in objective responses in 16 of 22 (73%) of patients treated, of whom 11 had recurrent disease. Two of these were complete responses, one of which was confirmed pathologically [Forastiere et al, 1987]. Even in metastatic malignant melanoma, a tumour resistant to most forms of chemotherapy, high dose cisplatin administered with a normal tissue protecting thiophosphate WR2721 achieved response rates of 53% although the median duration of response was short at 4 months [Glover et al, 1987].

The effect of single agent high dose cisplatin (up to 140mg/m^2) in lung cancer was disappointing, with only 1 of 9 patients with small cell cancer (SCLC) and none of the 24 patients with non-small cell cancer (NSCLC) achieving a response [Bhuchar and Lanzotti, 1982]. Twenty patients had received previous chemotherapy and this may have had some bearing on the poor response rates.

In children with solid tumours, delivery of high dose cisplatin (40mg/m^2 daily x5) by continuous infusion resulted in progressive enhancement of tissue exposure to the free cytotoxic drug but increased toxicity was experienced and there was a concomitant decrease in therapeutic effect [Dominici et al, 1989].

High dose platinum has also been delivered by alternative routes with the aim of increasing the local tumour concentration of the drug while minimising the side effects induced in other organs, for example the kidneys. Intraperitoneal infusion of high dose cisplatin (200mg/m^2) with etoposide and kidney-protecting sodium thiosulphate was given to 37 patients with ovarian cancer who had failed primary intravenous cisplatin-based treatment. The overall response rate was 65% and 9/24 patients had a pathological complete response (CR). Median survival was 26 months from the start of ip therapy. As primary treatment in 23 patients with stage III/IV ovarian cancer, the response rate was 14/23 and 43% had a pathological CR at second-look laparotomy after 6 treatments [Howell et al, 1991].

1.1c Toxicity of cisplatin.

The main problem encountered with cisplatin is the severe toxicity which continues to limit its clinical use. The frequency of nephrotoxicity is high, with 61% of courses of cisplatin given at 100mg/m^2 associated with acute renal toxicity [Rossof et al, 1972]. Subsequently, prehydration and mannitol diuresis were given before platinum and although the rate of delivery of cisplatin was 120mg/m^2 over 15 minutes, a protective effect was observed [Hayes et al, 1977]. The histological changes seen in the kidney after cisplatin treatment are focal acute tubular necrosis affecting primarily the distal collecting tubules and collecting ducts. Dilatation of the convoluted tubules and cast formation are also reported [Gonzales-Vitale et al, 1977].

The concurrent administration of aminoglycoside antibiotics has been found to increase the cisplatin-induced nephrotoxicity [Gonzales-Vitale et al, 1978] and this combination of drugs should be avoided whenever possible. The mechanism by which renal toxicity is produced has not yet been fully elucidated, but is partially due to the known fact that heavy metals induce passive backflow of glomerular filtrate across the disrupted tubular epithelium [Hollenberg, 1970]. The brush border of the proximal tubule is the principal site of toxicity induced by the heavy metals, and reduced glomerular filtration and renal blood flow to the cortex and the medulla mediated through the renin-angiotensin system result in acute renal failure [Madias and Harrington, 1978]. Increasing the volume of fluid traversing the renal tubules through mannitol, frusemide and saline diuresis of up to 2 litres in 6 hours can reduce the nephrotoxicity as explained above [Chary et al, 1977]. In the high dose cisplatin regimen of Corden and colleagues minimal nephrotoxicity was seen following 200mg/m^2 with 6l/day normal saline starting from 12 hours before starting cisplatin to 24 hours after completion of treatment [Corden et al, 1985]. With this regimen, the other toxic effects - detailed below - were at least equivalent to those seen with conventional cisplatin doses. It was postulated that the high concentration of the chloride ion in the prehydration fluid helped to protect the luminal cells from damage through mass action lowering of the reaction rate of cisplatin hydrolysis and by reducing reactions with tubular sulfhydryl-containing enzymes. Nephrotoxicity was measured only through estimation of the serum

creatinine and creatinine clearance in the study and it has since been shown that these parameters correlate poorly with the glomerular filtration rate (GFR) during platinum treatment, unlike the ^{51}Cr-EDTA clearance which is now used [Daugaard et al, 1988].

In a study where the nephrotoxicity of cisplatin in rats was reduced through delivery of the drug in a liposome carrier system, the antitumour effect of cisplatin was preserved at a dose of 2mg/kg, but tumour regrowth developed earlier than it would have done after delivery of free cisplatin [Steerenberg et al, 1988]. It would therefore seem that unlike the promising results obtained for encapsulation of adriamycin and methotrexate, liposomes are not a satisfactory carrier system for cisplatin.

Nausea and vomiting are other prominent side effects of cisplatin, and they may be very severe and protracted. These are toxicities which many patients find most distressing [Coates et al, 1983], and some people refuse further treatment even with potentially curative treatment because of the unacceptability of prolonged gastrointestinal upset. Even high doses of antiemetics such as metoclopramide (2mg/kg by intravenous injection), although superior in efficacy to phenothiazines and dexamethasone, fail to alleviate the nausea and vomiting produced by platinum chemotherapy [Cunningham et al, 1985]. The problems with akathisia and dystonic reactions experienced by patients who were treated with high dose

metoclopramide encouraged further research into the mechanisms of nausea and vomiting, and this resulted in the development of the highly selective 5-hydroxytryptamine-3 (5-HT_3) receptor antagonists which are effective antiemetics devoid of the adverse effects associated with dopamine receptor blockade. Initial results of trials with ondansetron, the first selective 5-HT_3 receptor antagonist, produced complete or major control in almost two thirds of platinum induced emesis when given prophylactically and in addition to the increased efficacy there was reduced toxicity reported by the patients [Marty et al, 1990]. Since 40% of patients suffer from acute or delayed vomiting despite use of 5-HT_3 antagonists, combination treatment with the addition of dexamethasone to the antiemetic regimen is recommended to increase efficacy prior to administration of platinum chemotherapy [Cunningham et al, 1989].

Bilateral, symmetrical high-tone deafness is also a well recognised side-effect of cisplatin chemotherapy, and is observed in up to 90% of patients receiving standard doses of up to 120mg/m^2 per course [Ellerby et al, 1974]. When the dose is increased to 200mg/m^2, 75% of patients receiving two courses and 92% who had three courses developed significant hearing loss with 83% suffering severe damage [Pollera et al, 1988]. The ototoxicity is most often experienced by people who already have hearing problems through disease or old age. It is dose-related, cumulative and irreversible [Helson et al, 1978], and treatment may have to be discontinued if it

develops. Neither the administration of hypertonic saline nor vigorous hydration - which are effective in minimising renal toxicity after cisplatin treatment - have any effect on ototoxicity. Often the earliest indication of ototoxicity is the development of tinnitus, and pre-treatment hearing problems or new development of tinnitus should alert the treating clinician to the need for formal assessment of the patient by audiometry. Hearing loss at 8000Hz is predictive of early deterioration and suggests that further cisplatin doses should be modified. Threshold evaluation in frequencies below 6000Hz indicates serious ototoxicity and in most cases platinum therapy should be discontinued in these cases.

Peripheral neuropathy is an additional and significant side-effect first reported by Kedar and colleagues following a total cisplatin dose of 500mg/m^2 [Kedar et al, 1978]. Damage is predominantly to the large sensory fibres with numbness and tingling as the first symptom. Later, proprioception is affected and a severe sensory ataxia can develop. Symptoms may begin and progress after treatment with cisplatin has been discontinued [Mollman, 1990]. The neuropathy appears to be dose dependent, usually appearing after a cumulative dose of 300-600mg/m^2. Recovery may take a year or more, consistent with the histopathological findings of segmental demyelination [Marin and Rierson, 1979]. The severity of the neurotoxicity induced by higher doses of cisplatin is such that it precludes their long term use [Bagley et al, 1985].

Another important toxic effect of cisplatin is that it alters gonadal function, gonadotrophin levels and steroid hormone levels in humans. Sterility may result from cisplatin treatment and this is a significant problem when one considers the young age of many men treated for testicular cancer. The testosterone depletion seen in adult male rats after cisplatin treatment is most likely to be due to its effects on hepatic enzyme expression, specifically on the hepatic p450 system [LeBlanc et al, 1992].

1.1d Prevention of cisplatin toxicity.

Attempts have been made to find compounds which may be given in conjunction with cisplatin to reduce its toxicity. The introduction of prehydration, diuretics and chloride loading to protect renal function has been mentioned above. The concurrent administration of cisplatin given intraperitoneally and sodium thiosulphate administered intravenously was originally thought to reduce the nephrotoxicity of treatment [Howell et al, 1983] but further investigation of the combination in animal models resulted in the conclusion that the thiosulphate reduced the antitumour activity of the cisplatin when they were given together [Aamdal et al, 1988]. The modulating agent WR 2721 is a prodrug of the radioprotective thiol compound WR1065. It has been shown to protect mice from the nephrotoxic and myelotoxic action of cisplatin without compromising its antitumour effect [Yuhas et al, 1980]. It has been proposed from study of the *in vitro* model system that cisplatin induced DNA damage can be

prevented by tissues first accumulating WR1065. In addition, WR1065 is thought to increase enzymatic DNA repair through its effect on conformational changes in DNA, so post cisplatin administration of WR2721 may also have a protective effect [Treskes et al, 1992].

Considering avoidance of neurotoxicity, a report by van der Hoop and colleagues stated that an analogue of ACTH (Org 2766) was effective in preventing neurotoxicity in 39 evaluable women with ovarian cancer who were treated with cumulative doses of 494mg/m^2 [van der Hoop et al, 1990] . Further trials are required to confirm the results and to ensure that the combination of drugs does not adversely affect cisplatin's antitumour activity.

The introduction to the clinic of the 5-HT_3 antagonist group of antiemetics has significantly reduced the problem of cisplatin-induced nausea and vomiting, as discussed above.

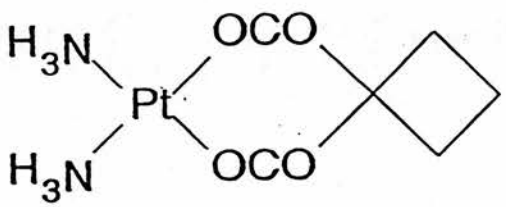
The fact remains that even if it were possible to minimise the many toxic side-effects of cisplatin, the problem of resistance to the drug - either primary or acquired - would continue to limit its therapeutic application. The best way by which to circumvent these disadvantages may therefore be to develop analogues of cisplatin which are at least equally effective in the clinic but which induce fewer and less serious side-effects. This aim was

partially achieved by the synthesis of carboplatin which is discussed below.

1.2 The development of carboplatin.

The need for the development of an analogue of cisplatin has been made clear by the problems outlined above. It was in 1978 that carboplatin (cis-diammine-1,1-cyclobutane dicarboxylate platinum II, CBDCA, JM8) was synthesised (structure Figure 1.3) by Cleare and colleagues [Cleare, 1978]. Preclinical trials identified it as an agent which had efficacy similar to that of cisplatin with a more acceptable toxicity profile [Harrap, 1985]. The mechanism of action of carboplatin is believed to be the same as that of cisplatin in that it too produces DNA inter- and intra-strand cross links. The rates of reaction of the arms of carboplatin are significantly different, unlike the case for cisplatin. Carboplatin induces formation of interstrand cross-links, and equal binding of the two compounds to cellular DNA in culture was shown to produce equivalent cytotoxicity [Knox et al, 1986]. The cyclobutane-dicarboxylato bidentate structure of carboplatin is more soluble but less reactive than the chloride of cisplatin, making the carboplatin more chemically stable than the parent analogue and this results in a larger dose of carboplatin being required to achieve the same degree of cytotoxicity [Knox et al, 1986].

FIGURE 1.3 STRUCTURE OF CARBOPLATIN



Carboplatin

1.2a Clinical activity of carboplatin.

There have been criticisms that the individual role of carboplatin as an antitumour agent has not been specifically determined [Von Hoff, 1987], but it has been established in several studies that it demonstrates at least equivalent activity to cisplatin in the treatment of ovarian cancer [Advanced Ovarian Cancer Trialists Group: 1991]. One paper which described in detail the toxicity and efficacy of cisplatin and carboplatin in advanced ovarian cancer was published by Adams [Adams et al, 1989]. The clinical response (CR + PR) to cisplatin was 19/40 patients compared with 27/40 for carboplatin but there was no difference in two year survival between the two patient groups. The toxicity of carboplatin was certainly less, with the median number of vomiting episodes per cycle being eight times more for cisplatin than for carboplatin. WHO Grade I renal toxicity was seen in 67.5% of the cisplatin treated patients compared with 2.5% of the recipients of carboplatin (WHO, 1979). With the use of standard doses of carboplatin, neither ototoxicity nor neuropathy was witnessed whereas 45% of the cisplatin treated patients had evidence of hearing damage at audiometry, and 22.5% of the cisplatin treated patients had WHO grade I neurotoxicity. The reduction in renal toxicity following carboplatin as opposed to cisplatin treatment allows repeated dosing and enables it to be given to "salvage" patients who may have responded to cisplatin but in whom deterioration in renal function has precluded further treatment. Intraperitoneal carboplatin 300mg/m^2 was delivered to patients with ovarian

cancer who had macroscopic disease at second look laparotomy following iv carboplatin. The response rate was lower than expected at 12% in the 25 evaluable patients [Guastalla et al, 1994].

The single agent usefulness of carboplatin in small cell lung cancer is impressive with a 60% response rate in chemo-naive patients [Smith and Evans, 1985]. This response rate can not be related accurately to results obtained with cisplatin since the latter was not tested in a comparative manner. Carboplatin and etoposide in combination as first-line therapy for small cell lung cancer have been shown to achieve an objective response rate of 85% in a study involving 52 patients, but as with other combinations the initial response rates did not translate into improved response duration (6 months in this study) or survival (9.5 months) [Smith et al, 1987]. Results for combination therapy with cisplatin and etoposide in the same tumour were comparable at 86%, but nephrotoxicity occurred in 15/31 patients treated with cisplatin, a toxicity not seen in the carboplatin treated patients [Evans et al, 1985]. Non small cell lung cancer has been treated with carboplatin alone and in combination with cisplatin to exploit the differential pharmacodynamic and dose limiting toxicities of the drugs. No difference in efficacy was seen when the drugs were combined as first line chemotherapy and delivered monthly to 76 patients. The overall response rate was 13% in the 68 evaluable patients [Kreisman et al, 1990].

The frequent pre-existing renal damage in patients with bladder cancer make

carboplatin an attractive alternative to the active cisplatin. However, the effectiveness of carboplatin was less with only 3 partial responses (PR's) in 48 patients with stage IV disease [MRC Working Party in Urological Cancer, 1987].

In head and neck cancer, the single agent response rate with carboplatin was ranged from 14 to 28% [Canetta et al, 1988] but combination with other agents (eg. 5-Fluorouracil and methotrexate) may enhance the response rate and results of trials are awaited.

In seminomatous tumours treatment with single agent carboplatin has been shown to achieve very high response rates which translate into 2-year survival rates of over 90%. In a study reported by Horwich and colleagues, 34 chemo-naive patients with advanced disease were treated with single agent carboplatin 400mg/m^2 at 4 weekly intervals. The 94% actuarial 2-year survival rate was equivalent to that seen with aggressive combination regimens and toxicity was significantly less (Horwich et al, 1989].

The documented activity of carboplatin in cervical cancer is equivalent to that of cisplatin (response rate 12/46 or 26%) and toxicity in the Phase II study was certainly reduced (Lira-Puerto et al, 1991). In advanced endometrial cancer 28% of patients achieved a PR with median response duration 129 days [Long et al, 1988].

In advanced gastric cancer, the single agent activity of carboplatin in a phase II study of 23 patients was 9% with dose schedule 130mg/m^2 on days 1,3 and 5 of a 28 day cycle [Preusser et al, 1990]. The drug was well tolerated with the only significant toxicity being haematological. The duration of response was only 4 months and 11/20 patients subsequently responded to second line chemotherapy.

A review by Smith and Talbot stated that single agent carboplatin in breast cancer achieved a response rate of 28% in previously untreated patients with advanced disease [Smith and Talbot, 1992]. Predictably, the response was significantly worse in previously treated patients, reaching only 3%. There are only limited data on the use of carboplatin in combination treatment for breast cancer, but the estimated response rate ranges from 25% to 44% in a pretreated patient population. High dose carboplatin treatment in combination with thiotepa and cyclophosphamide resulted in a response rate of 81% in a study involving 16 patients [Eder et al, 1990].

Prolonged responses have been reported in patients with ovarian cancer who have undergone protracted treatment with carboplatin, raising the possibility that response duration may be increased if the optimal duration of treatment can be established [Muggia, 1989]. One of the advantages of the reduced toxicity of carboplatin compared with cisplatin is that more protracted treatment is feasible and "rescue" is possible for patients with responding

tumours who have unacceptable side-effects of cisplatin.

1.2b Toxicity of carboplatin.

Carboplatin is certainly less toxic than its predecessor cisplatin. Dose limiting toxicity is myelosuppression [Calvert et al, 1982; Rose and Shurig, 1985]. There is a much smaller incidence of nephrotoxicity [Wiltshaw, 1985]. Sterility is not a problem encountered with carboplatin in contrast to the previously mentioned effects of cisplatin [LeBlanc et al, 1992].

Pharmacokinetically, carboplatin is handled by the kidney in such a way that its clearance approaches the GFR. This enables easier calculation of the appropriate dose to be prescribed to avoid excessive myelotoxicity [Egorin et al, 1984] and it can be used prospectively [Egorin et al, 1985]. The original formula correlated platelet nadir (expressed as a percentage of the pretreatment count) with the free platinum concentration/time data (area under the curve or AUC in mg/ml x minutes) and required knowledge of the pretreatment GFR, the body surface area and the patient's prior chemotherapy status. Later, Calvert developed a formula which provides a simple and consistent method of determining carboplatin dose in adults where the measure of drug exposure is AUC and not toxicity: total dose given (mg) $=(GFR+25)xAUC$ required. The advantage of this formula is that it is not influenced by previous or concurrent myelosuppressive therapy or supportive measures [Calvert et al, 1989]. In addition, the formula allows

calculation of the constant carboplatin exposure time when the drug is given in combination and facilitates the estimation of actual dose given.

1.2c High dose carboplatin.

Several studies have been published where carboplatin has been administered in doses higher than the conventional 400mg/m^2 or AUC 6mg/ml x minutes. It was predicted that high dose carboplatin would result in less severe toxicity than high doses of cisplatin and that myelosuppression would be the main limitation to dose incrementation.

Carboplatin in doses of 800 to 1600mg/m_2 were delivered to patients with lung cancer or mesothelioma without bone marrow rescue. A total of 16 patients were treated with 5 to 10 cycles of chemotherapy at 800, 1200 or 1600mg/m^2 with no intra-patient dose elevation [Gore et al, 1987]. Myelosuppression was the main toxicity and at the top dose the Grade IV thrombocytopenia (from day 12) lasted from 8 to 33 days. Grade IV neutropenia was of shorter duration (8 to 14 days). Ototoxicity was documented in 3 patients at doses over 800mg/m^2 and a mild peripheral neuropathy was recorded in 3 patients, also at the higher dose levels.

In a study where carboplatin 300mg/m^2 daily x5 was given to treat acute leukaemia, ototoxicity was the dose limiting factor while continuous infusion of 265mg/m^2 x5 days resulted in myelosupression and alteration of liver

function. It was postulated in the same study that the concomitant administration of aminoglycoside antibiotics might have been the factor that accounted for the difference in otoxicity witnessed [Lee et al, 1987; Meyers et al, 1987].

More recently, haematopoietic growth factors were given concurrently with infusions of autologous peripheral blood progenitor cells (PBPC) to reduce the duration of myelosuppression from high dose carboplatin. There was a significant reduction in the duration of neutropenia and thrombocytopenia in the 18 treated patients when PBPC support was added to carboplatin and granulocyte-macrophage colony stimulating factor (GM-CSF) therapy for the second 20 (of a total of 40) courses of carboplatin at dose 1200mg/m^2 over 96 hours. The main aim of the trial was to ascertain and subsequently alleviate the toxicity of the regimen, but a 70% response rate was seen in the previously treated group of patients. The 3 complete responses (CR's) were in patients with ovarian cancer. A 57% increase in the total dose of drug was delivered to the patients who had haematopoietic growth factors and PBPC [Shea et al, 1992]. These data are encouraging for those oncologists who are currently investigating the role of dose intensity with PBSC rescue in several solid tumour types.

Currently a study of high versus conventional dose carboplatin in initial

treatment of advanced ovarian cancer is in progress at the Royal Marsden Hospital. Using Calvert's formula incorporating the glomerular filtration rate as measured by ^{51}Cr-EDTA clearance to achieve a known AUC representative of drug exposure time [Calvert et al, 1989], patients are randomised to receive carboplatin doses to achieve either AUC6 or AUC 12mg/ml x minutes. Those patients randomised to the high dose arm receive a total of four treatments at monthly intervals compared with six monthly treatments delivered to those in the conventional dose arm of the study. The results should test the contention of Jodrell and colleagues that an increase of AUC from 5 to 7mg/ml x minutes will result in an increase in toxicity without associated increase in response [Jodrell et al, 1992].

1.3 Resistance to cisplatin and carboplatin.

Apart from the toxicity of cisplatin and carboplatin, the other problem that severely limits their use in the clinic is the development of resistance. Even in advanced ovarian cancer where the platinum-based drugs have produced high complete response rates, prolongation of disease free survival and statistically significant prolongation of overall survival, the majority of patients will relapse. The likelihood of responding to a rechallenge with platinum depends on the duration of response to the original treatment. In a retrospective analysis of responses in patients with ovarian cancer who were included in phase II studies, there was a significant increase in the chance of a drug inducing an antitumour response if the patient had

completed initial treatment more than six months previously and had responded to it [Blackledge et al, 1989]. The other factor which was shown to be of relevance was the FIGO staging of the patient, with more advanced disease predicting negatively for response. The importance of duration of response to the initial chemotherapy treatment was supported by the findings of Gore and colleagues who reported the response to a platinum rechallenge as 57% if the original response lasts for more than two years compared with 17% if progressive disease were detected less than 12 months from the first treatment [Gore et al, 1990].

Many different groups have investigated the mechanisms of resistance development with the eventual aim of finding a means by which to reverse or to overcome them. Attempts to predict the likelihood of a patient surviving long term after an initial response to cisplatin have included study of short-term in vitro chemosensitivity of tumour samples obtained at surgery. One study involving 61 patients with stage III ovarian cancer reported a positive predictive value of 72% and a negative value of 66% in terms of a definite clinical outcome - survival or death - using an assay of cisplatin-induced inhibition of cell proliferation [Khoo et al, 1988]. This approach does not, however, consider the reasons for eventual relapse and demise of the patient and is therefore of limited use in clinical practice.

There are several ways in which malignant cells may develop resistance to

platinum chemotherapy. The mechanisms which have been investigated are outlined below.

1.3a Studies of reduced accumulation.

Several authors have suggested that decreased accumulation of drug is a mechanism of platinum resitance present in several different cell lines *in vitro* [Eichholtz-Wirth and Hietel, 1986; Hromas et al, 1987a; Waud, 1987; Richon et al, 1987; Kraker and Moore, 1988; Andrews et al, 1988a]. The lack of evidence for increased efflux of drug suggests that the mechanism of resistance to platinum in these cells may differ from that found in mouse fibroblast NIH 3T3 cells which have been transformed with *ras* oncogenes. The protein encoded by *ras*, was located on the inner surface of the cell membrane and was associated with increased cisplatin resistance in these cells. The postulate was that it may increase the cells' ability to pump out cisplatin [Sklar, 1988].

An example where increased drug efflux contributes to resistance is that found in neoplastic cells which are resistant to adriamycin, vinblastine, actinomycin D and colchine. Multiple drug resistance (MDR) is known to be associated with increased expression of the P170 P-glycoprotein (where P represents permeability), which is encoded by the mdr^1 gene and enhances the cells' ability to pump out the drugs [Inaba et al, 1979; Kartner et al, 1983]. To date, no evidence has been found to suggest that resistance to the platinum drugs depends on a similar mechanism.

On finding that the cisplatin resistant murine leukaemia cell line (L1210/cisplatin) bound 56% less platinum on its membrane than did the sensitive L1210, Gross and Scanlon initiated a more detailed study of the membrane of resistant cells. They concluded that resistance was associated with a change in the transport of neutral amino acids. The L1210/cisplatin cells under study were found to have a much lower requirement for exogenous methionine and a decreased methionine transport associated with decreased threonine uptake [Gross and Scanlon, 1986]. Shionoya and colleagues confirmed that this was also the case in the platinum resistant K562 chronic myeloid leukaemia cell line [Shionoya and Scanlon, 1986]. These results confirm that there is a change at the membrane level in L1210/cisplatin cells, but more detailed study of the lipid content, surface membrane fluidity and temperature dependence of cisplatin accumulation in sensitive and resistant human ovarian cancer cells showed that there was no significant difference in the basic membrane structure between the two cell lines [Mann et al, 1988]. Previous studies reported that cisplatin accumulation is modulated by cAMP and depends on the membrane potential [Andrews et al, 1988a] but more recently, Andrews and colleagues have concluded that the Na^+,K^+-ATPase plays a central role in the cellular uptake of cisplatin since inhibition of the Na^+,K^+-ATPase by ouabain dissipates the Na^+ gradient and directly affects cisplatin influx [Andrews et al, 1991].

It was reported in 1981 that the coadministration of verapamil, a calcium channel blocker, with the anticancer drugs vincristine and vinblastine led to an increase in cytotoxicity [Tsuruo et al, 1981] . A similar experiment was then carried out to investigate the effect of administering cisplatin and verapamil to Balb/c athymic mice bearing a transplanted human neuroblastoma. The addition of verapamil was found to increase the antitumour effect of cisplatin in this tumour [Ikeda et al, 1987], but it was a different type of calcium channel blocker (nifedipine - dihydropyridine class) that caused enhancement of antitumour activity in an inherently cisplatin resistant Lewis lung carcinoma line [Onada et al, 1988]. There is no evidence to date that calcium channel blockers increase uptake or decrease efflux of cisplatin, but the fact remains that the important role of the cell membrane in drug resistance is again emphasised by these findings.

1.3b The role of glutathione.

Glutathione is a tripeptide thiol found in virtually all cells at millimolar concentrations, levels substantially higher than many other intracellular molecules. Its role in cellular resistance was elucidated in the study of resistant Ehrlich ascites tumour sublines reported by Revesz and colleagues [Revesz et al, 1963]. All but one of the sublines which had resistance induced by sublethal *in vitro* irradiation between passages contained an increased concentration of non-protein sulfhydryl and were more resistant

than the parent Ehrlich cell line to the growth inhibitory effect of 20Gy radiation in nitrogen. In the presence of oxygen, all sublines were as susceptible to the effects of radiation as the parent line. The theory accepted as an explanation for the difference in sensitivity between these cell lines was that GSH plays a role in detoxification of radiation-induced toxins such as free radicals and/or the repair of critically damaged cell structures. In addition to playing a role in cellular resistance to and repair following exposure to radiation, Calcutt and Connors in 1963 reported that the therapeutic response of some tumours to merophan, an isomer of melphalan, correlated with the ratio of protein-free to protein-bound sulfhydryl [Calcutt and Connors, 1963]. More recently Susukake and colleagues showed that an L1210 leukaemia line made resistant to melphalan contained a 2-fold greater concentration of GSH and could be resensitised by lowering its GSH content [Susukake et al, 1982]. The relationship between increased GSH and resistance both to radiation and to several antineoplastic drugs was reviewed by Arrick and Nathan [Arrick and Nathan, 1984]. It might have been expected that elevation in intracellular GSH concentration would play a part in resistance to platinum-based anticancer drugs because of the similarity in their action to that of the other alkylating agents.

Further to the discovery of the effects of increased glutathione, it was demonstrated that if the intracellular concentration were decreased through

prolonged incubation of the cells with the specific δ-glutamylcysteine synthetase inhibitor buthionine sulfoximine (BSO), the full sensitivity of the cells to cisplatin was recovered [Hromas et al, 1987b; Andrews et al, 1988b]. The mechanism by which GSH exerts its protective effect may be either through reaction between cisplatin and GSH preventing active electrophilic drug from reaching intracellular targets or, alternatively, GSH may act as an important mediator in the repair of the damage to DNA induced by platinum (Andrews PA et al, 1988b). Further research has revealed that depletion of GSH with BSO does inhibit DNA repair, and that treatment of the cells with glutathione ester after BSO exposure results in complete recovery of DNA repair activity [Lai et al, 1989]. It has been postulated by several researchers that the enzyme glutathione S-transferase (GST) is more important than the tripeptide itself in platinum resistance. The involvement of GST was first suspected when it was reported that resistance in an adriamycin resistant human breast cancer subline was associated with a 45-fold increase in GST activity [Batist et al, 1986]. However, no correlation between total GST activity and drug sensitivity was measured in a detailed study of 14 parent and 15 drug- and X-ray selected resistant sublines published by Hosking and colleagues [Hosking et al, 1990]. Modified GSTs have been identified in a range of multidrug resistant tumour sublines but it is now thought that it is not the total GST that is important, rather the differences in isoenzyme composition may determine the development of a particular phenotype of resistance [Sato,

1989].

1.3c Metallothioneins.

Metallothioneins are intracellular proteins rich in sulfhydryl groups (composed of 30% cysteine) and they are believed to play a central role in metabolism and storage of heavy metals in the body [Webb, 1972]. It has been shown that cells which have a high concentration of metallothionein are resistant to cisplatin [Bakka et al, 1981]. Although the initial observations were made in cells which had been made resistant to cadmium, elevated metallothionein levels have now been found in a cisplatin resistant head and neck tumour [Teicher et al, 1987] and a prostate tumour line [Metcalfe et al, 1986]. Cisplatin resistant ovarian cancer cells have also been shown to contain an elevated concentration of metallothionein, but *in vitro* selection with cisplatin did not increase the levels of these proteins [Andrews et al, 1987a]. It may be that further investigation of the role of metallothionein in drug resistance will offer a mechanism by which platinum resistance may be overcome, but the first reports exploiting the results have concentrated on the induction of metallothionein in the kidney using bismuth subnitrate (BSN). Increased levels of metallothionein induced by this method have been found to reduce the toxic effects of cisplatin without affecting its antitumour activity [Satoh et al, 1988]. More recently, studies on an L1210 cell line and its cisplatin resistant variant (L1210-PDD) have shown that both lines contain metallothioneins which are inducible by exposure to zinc *in*

vitro. Zinc induction of metallothioneins was less effective in the L1210-PPD line which showed no cross resistance to zinc and a heightened sensitivity to cadmium. L1210 cells were found to produce metallothioneins in response to cisplatin whereas the L1210-PPD did not. The pattern suggested that enhanced synthesis of metallothioneins was not the mechanism by which L1210-PPD cells were resistant to cisplatin cytotoxicity [Farnworth et al, 1990].

1.3d Topoisomerase.

There is ample evidence to support the theory that platinum-DNA inter- and intrastrand crosslinking interfere with replication of tumour cells (see section 1.1a above). It is possible that increased repair of the damaged areas may confer resistance to platinum based drugs. DNA topoisomerases play a major role in DNA repair, with topoisomerase II thought to be the more important enzyme [Downes et al, 1988]. The adducts may become more accessible to repair enzymes via alterations in the topology of DNA. The interaction of cisplatin with drugs that affect topoisomerase was investigated by Katz and colleagues using the human ovarian cancer cell line 2008 [Katz et al, 1990]. There was evidence of additive interaction between cisplatin and topoisomerase I modulators camptothecin and B-lapachone and between cisplatin and the topoisomerase II modulator novobiocin. There was antagonism noted between cisplatin and the topoisomerase II inhibitor etoposide despite the known clinical synergy of

these two drugs.

The conclusion to be drawn from all of these experiments must be that there is no single mechanism that imparts to malignant cells the property of resistance to the platinum anticancer drugs. Innate and secondary resistance is mediated through a combination of factors therefore the search for agents which will reverse the resistance or for alternative platinum-based chemotherapy will continue.

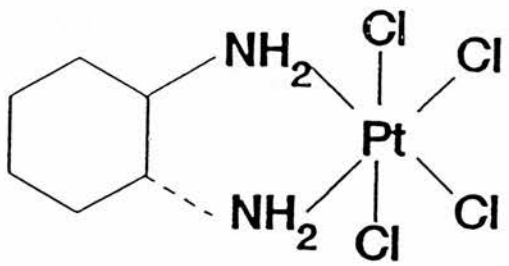
1.4 Studies of novel platinum-based drugs.

Despite advances in treatment of tumours with ever increasing doses of cisplatin and carboplatin and continued investigation of the efficacy of combination chemotherapy in the hope that a synchronous or even additive effect in antitumour activity may be discovered, one of the main thrusts of research in cancer treatment is the search for novel platinum based drugs. The aim is to discover one or more agents which have a broader spectrum of activity coupled with less severe toxic side-effects. In addition to these properties, ease of solubility and of formulation are essential features if a drug is to become a viable antineoplastic treatment. The new platinum compounds and their performance in phase I and II trials are discussed below.

1.4a Tetraplatin.

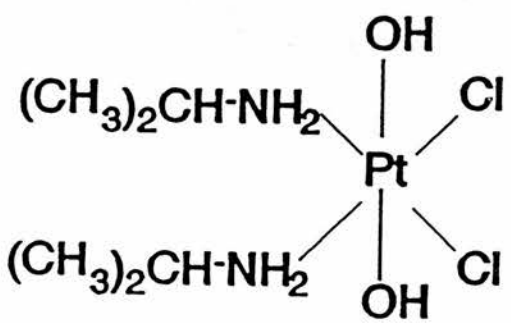
The objective behind the development of tetraplatin (d,l-trans-isomeric 1,2-diaminocyclohexane tetrachloroplatinum (IV); ormaplatin - structure Figure 1.4) was to produce a platinum complex which demonstrated activity against cisplatin resistant tumour cells and demonstrated suitable pharmaceutical properties for easy formulation. Tetraplatin was found to fulfil these criteria, showing activity superior to cisplatin against the P388/cisplatin, the intraperitoneally (ip) implanted murine L1210 leukaemia and other tumours *in vitro* [Anderson et al, 1986]. However, although tetraplatin retained activity against the cisplatin-resistant L1210, it was inactive against a correspondingly resistant murine ADJ/PC6 line [Goddard et al, 1991]. In a phase I clinical trials of tetraplatin with administration of drug on a day 1 and 8 schedule, the dose delivered ranged from 4.4 to 60.8mg/m^2 [Schilder et al, 1994]. Although nausea and vomiting equal to or greater than grade II was experienced by 40% of patients, it was said to be well controlled with standard antiemetics. A delayed, severe neurotoxicity was seen in 4 patients and was associated with cumulative doses above 200mg/m^2. One case of nephrotoxicity was also reported. The fact that the trial was closed without selection of a suitable dose for phase II studies suggests that the neurotoxicity witnessed in the phase I trial will be seriously detrimental to further development of tetraplatin.

FIGURE 1.4



Tetraplatin

FIGURE 1.5



Iproplatin (CHIP, JM9)

1.4b Iproplatin.

The cis-dichloro-trans-dihydroxy-bis-isopropylamine platinum IV compound (CHIP, JM9 - Figure 1.5) was developed in 1978 and was found to be active against the L1210 tumour and against plasmacytoma PC6 [Cleare et al, 1978]. Bramwell and colleagues reported on treatment of advanced ovarian cancer in 28 patients treated with Iproplatin in a phase I-II trial. Six complete and 2 partial responses were achieved in 10 patients who had received no prior chemotherapy, and 1 complete and 3 partial responses were seen in 16 patients who had previously been treated with alkylating agents [Bramwell et al, 1985]. Drug doses employed in the ovarian trial were from 180mg/m^2 to 350mg/m^2 in three increments. Twenty-three patients had undergone previous chemotherapy treatments. Responding patients received a total of six courses of chemotherapy with Iproplatin. There was no significant renal impairment, electrolyte imbalance, hearing loss or peripheral neuropathy. The dose-limiting toxic effect was thrombocytopenia which was dose related and cumulative. Neutropenia and anaemia were also seen with 14 of the 39 patients in the trial requiring transfusion. Gastrointestinal upset with explosive diarrhoea was a severe and distressing side-effect of iproplatin treatment.

In squamous carcinoma of the head and neck, no antitumour effect was seen in any of the 50 patients treated with Iproplatin 240-300mg/m^2 every four weeks for a total of 101 four week courses [Abele et al, 1987].

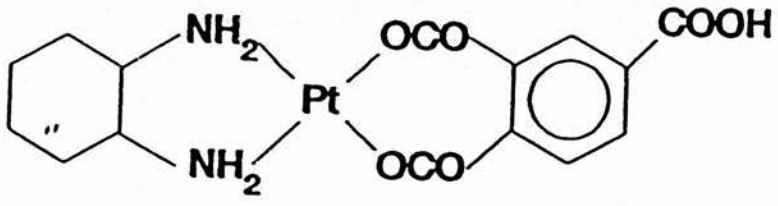
Iproplatin was shown to result in a response rate of only 8% (1/29) of

patients with breast cancer when delivered at 45mg/m^2 daily for 5 days every month [Meisner et al, 1989]. All patients had received previous chemotherapy.

1.4c DACCP.

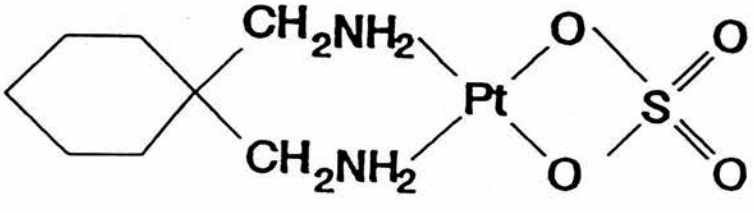
DACCP (JM82 - structure Figure 1.6) is another of the diaminocyclohexane analogues in which interest was initially shown because of its activity in L1210 lines resistant to cisplatin [Burchenal et al, 1978]. The major disadvantage of the drug was its restricted solubility. Phase I studies revealed the dose-limiting toxicity to be thrombocytopenia. Renal toxicity was seen at the higher dose levels of DACCP. Neurotoxicity was also reported but nausea and vomiting were not unacceptably severe. Of the tumours studied - testicular, ovarian, bladder, oesophagus, stomach, lung and colon - no advantage was seen over cisplatin therapy, although some of the patients had been extensively pretreated [Kelsen et al, 1982]. No activity was found in a phase II trial of DACCP in refractory germ cell tumours [Chun et al, 1985]. With formulation problems prominent and without any evidence of a therapeutic advantage in using DACCP, the prospect of further trials with it would appear to be remote.

FIGURE 1.6



JM82 (DACCP)

FIGURE 1.7



TN06

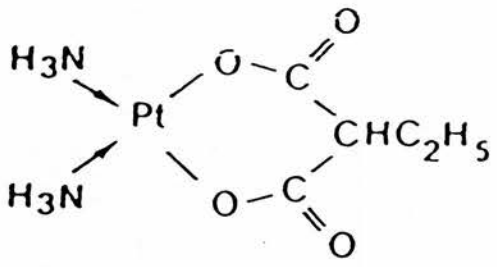
1.4d TNO6.

The 1,1-diaminomethylcyclohexane sulfatoplatinum (TNO6, Spiroplatin - structure shown Figure 1.7) was initially thought to be less nephrotoxic than cisplatin, but further studies revealed that severe, irreversible, occasionally late onset and often fatal renal failure developed after treatment, and this made its further investigation unacceptable [Offerman et al, 1985; Colombo et al, 1986; Van der Vijgh et al, 1988]. The extreme nausea and vomiting it induced also mitigated against further clinical application of TNO6 which was, in any case, apparently devoid of significant clinical activity.

1.4e JM40.

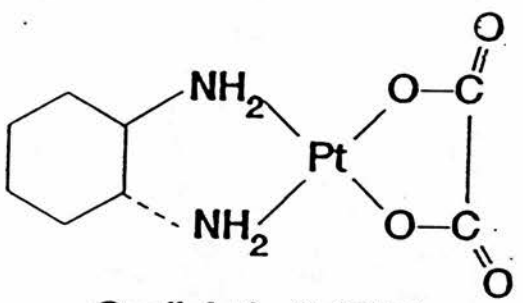
No phase II studies of ethylenediamine platinum II malonate (JM40 - structure Figure 1.8) have yet been published, but its reported toxicity in phase I was less severe than that of TNO6 or JM82. Nausea and vomiting were again the main dose-limiting side effects. Although nephrotoxicity occurred, it appeared to be reversible and myelosupression was not documented. The neuropathy reported by several patients treated with JM40 was not severe. Antitumour activity was present, with 2 PR's in the phase I study but this and toxicity will be studied more carefully if JM40 undergoes further clinical evaluation [Winograd et al, 1986].

FIGURE 1.8



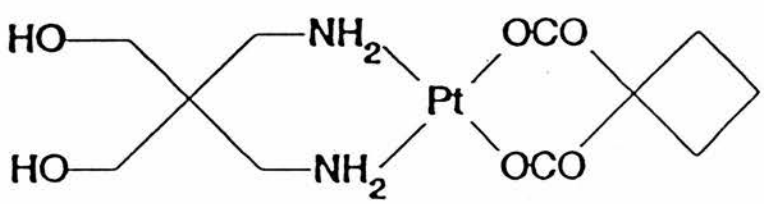
JM40

FIGURE 1.9



Oxaliplatin (l-OHP)

FIGURE 1.10



Zeniplatin (CL286, 558)

1.4f Oxaliplatin.

A phase I report published on oxaliplatin (trans-1-diaminocyclohexane oxalatoplatinum, 1-OHP; structure Figure 1.9) reported 4 partial responses in 44 patients. Doses ranged from 45 - 200mg/m^2 [Extra et al, 1990]. Although two of the responses were seen in oesophogeal cancer which is usually resistant to single agent chemotherapy, the toxicity induced by the drug was very concerning. The dose limiting toxicity was neuropathy which was first seen at a dose of 135mg/m^2 and continued thereafter, occurring in 75% of the courses. Six patients developed Grade III (WHO) neuropathy after a cumulative dose of 500mg, and it was stated that a long-lasting sensory neuropathy might result from treatment with the drug. The phase II studies have shown encouraging activity in melanoma and gastric tumours (overall response 5/16 and 5/8 respectively) and oxaliplatin will be studied further [Christian, 1992].

1.4g Zeniplatin.

Phase I studies with 2,2-bis(aminomethyl)-1,3-propanediol-N,M' 1,1cyclobutanedicarboxylato (2-)-0,0' platinum (Zeniplatin, Figure 1.10), a third generation platinum compound, reported that the dose-limiting toxicity was myelosuppression, especially leucopenia in heavily pretreated patients. Nausea and vomiting were also significant, and grade 2 alopecia, mucositis and diarrhoea were also seen. There was no ototoxicity or consistent change in renal function [Dodion et al, 1989]. Phase II studies with the drug

in ovarian carcinoma reported an overall response rate of 16% in a population of 23 evaluable patients. The median progression-free survival in responding patients was 11 months with overall survival 81% compared with survival of 9 months in patients with progressive disease. Zeniplatin had no direct effect on renal function as measured by isotope clearance, but one patient who was treated on a "marginal" creatinine clearance suffered from sudden and severe renal failure (Willemse et al, 1993). The Phase II studies in non-small cell lung cancer showed a response rate of 33% in the 18 assessable patients, but again nephrotoxicity was a problem and will seriously limit the further development of the drug [Jones and Smith, 1991].

1.5 Screening for novel platinum drugs.

Much time, effort and money have been spent on attempts to improve on cisplatin, and although carboplatin now has an important clinical role, the search goes on to develop related antitumour drugs which are less toxic and possibly more potent. In addition to improving the quality of response to platinum drugs, the aim is also to identify agents which will demonstrate antitumour activity against those cancers which have so far been resistant to all forms of chemotherapy. Compounds which are to be tested for oncological activity undergo very strict selection criteria prior to their introduction to clinical trials, and the approach to screening varies in different drug development centres.

At the National Cancer Institute (NCI) in America, the Cancer Chemotherapy National Service Centre (CCNSC) was established in 1955. The index tumours used in screening coumounds were the L1210 and P388 murine leukaemias, but a valid criticism of the drugs selected by this means was that they were effective mainly against human leukaemia and lymphoma but were of little use in treatment of more slowly growing solid tumours. Between 1975 and 1985, the sceening panel was changed in that animal solid tumours and human xenografts were developed to provide a second stage of drug selection, but the earlier problem of the type of drug found to be active in the pre-screen remained.

From 1985, the NCI decided to introduce human tumour cell lines with an in vitro assay as the primary cancer screen. The emphasis of drug screening was therefore changed to a disease-orientated approach with cell lines comprising a total of sixty different tumours - lung, colon, melanoma, renal, ovarian, brain and leukaemia. The current arrangement is that each chemical agent is tested in various concentrations against a wide variety of human tumours of both solid and haematological type. The system is now capable of testing over 1 000 extracts every week, and since the full cancer screen has been operational (April 1990) more than 27 000 defined chemical entities and thousands of crude natural product extracts have passed through the system. Only 4% of the screened compounds have been selected for secondary *in vitro* and *in vivo* evaluation [Grever et al, 1992].

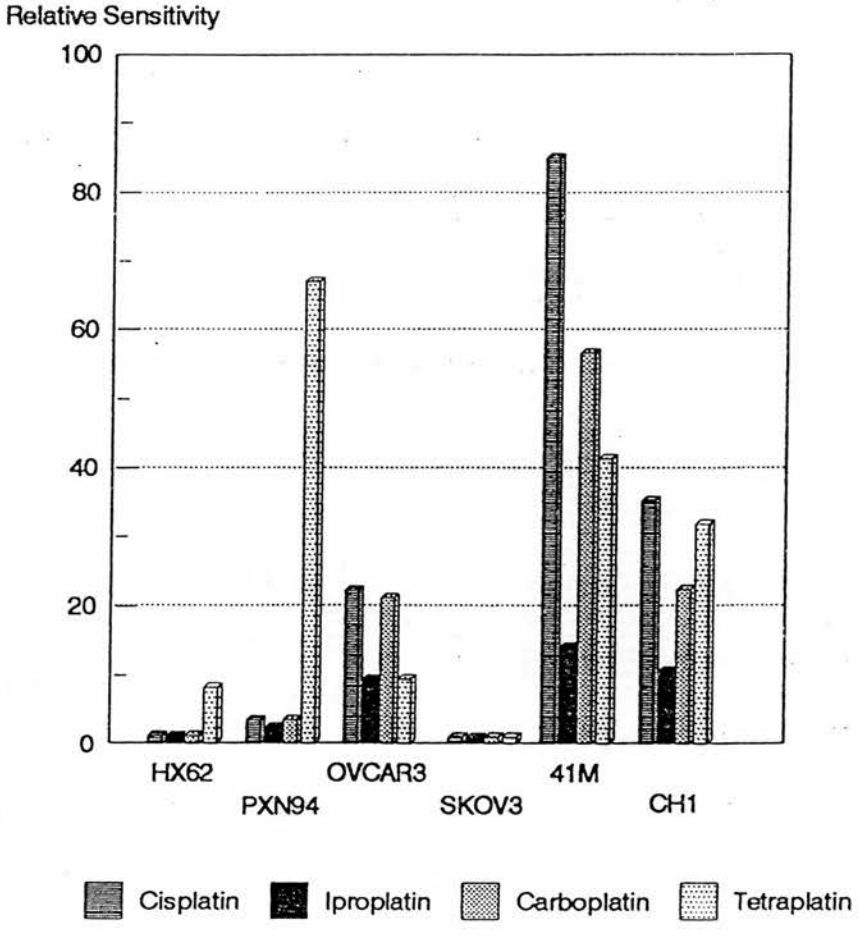
At the Institute of Cancer Research in Sutton (ICR), carboplatin was selected for further investigation mainly on the basis of its activity against the ADJ/PC6 murine plasmacytoma and the P246 human bronchogenic carcinoma xenografted into immune-deprived mice [Harrap, 1985]. The ADJ/PC6 provides the primary *in vivo* rodent screen for the identification of active novel platinum containing compounds while the L1210 murine leukaemia is used on an *ad hoc* basis. For both PC6 and L1210 tumours, resistant variants of the parent cell line have been developed through controlled exposure of the sensitive cells to cisplatin, carboplatin amd tetraplatin.

In the case of L1210, the resistant lines are designated L1210/cisplatin, L1210/carboplatin and L1210/tetraplatin. Details of the development of resistance are given later in the thesis. Another important feature of these murine lines at the ICR is that they are all freely transferable between the in vivo and the *in vitro* state thus enabling biochemical and pharmacological studies to be carried out. This feature is useful in the investigation of resistance development in tumours and provides an invaluable extension to the drug screening panel.

A more recent addition to the bank of cell lines at the ICR are the human ovarian cancer tumours. Six of these lines (named HX62, PXN94, OVCAR3, 41M, CH1 and SKOV3) have been introduced to the screening panel

because of the differential sensitivity which they exhibit to several 'calibrating' platinum drugs - cisplatin, tetraplatin, carboplatin and iproplatin (Figure 1.11). Details of the origins of these six cell lines - five of which also grow as xenografts - are given in Chapter 5.4. Their importance in drug development is that they may provide a more predicitve model of tumour sensitivity in man.

Figure 1.11 Relative sensitivity of ovarian carcinoma cell lines (with SKOV-3 line = standard 1)



1.6 Thesis aims.

Although the discovery and development of platinum containing anti-cancer drugs has had a major impact on the successful treatment of many tumour types, efficacy of the drugs is seriously limited by toxicity and by the development of tumour resistance.

An initial means by which to investigate the problems (with a view to overcoming them), has been to develop resistance to cisplatin, carboplatin and tetraplatin in the L1210 murine leukaemia line *in vitro*. These variant lines have been characterised biologically and have provided a model for the study of biochemical mechanisms associated with resistance, for example drug uptake, glutathione content and DNA platination. The methods employed in resistance development and in investigating resistance mechanisms are detailed in the thesis.

In addition, the resistant models have been used to screen both platinum II and platinum IV novel compounds provided by the Johnson Matthey Technology Centre. The novel compounds investigated have either shown good clinical activity when administered systemically to animals bearing the ADJ/PC6 plasmacytoma, or form part of a structure-activity series. Studies with the resistant models form part of a programme to identify a third generation platinum drug which may overcome the problem of resistance to cisplatin and carboplatin.

The other priority of the platinum programme is to identify a compound suitable for oral administration in the clinic. A number of platinum IV dicarboxylates have been found to demonstrate superior activity against the ADJ/PC6 tumour *in vivo* when delivered orally rather than systemically. These novel compounds have been evaluated for *in vitro* cytotoxicities against the L1210 variant lines. Six "lead" compounds have also been selected from the *in vivo* antitumour studies for further evaluation of their cytotoxic effects against human ovarian cancer cells in culture. Detailed pharmacology and haematological toxicity information has been obtained using the six lead compounds in BALBc mice.

The final aim was to select one or two compounds for preclinical development in the oral programme, and the first of these is currently being evaluated in phase I clinical trials.

CHAPTER 2

THE DEVELOPMENT AND CHARACTERISATION OF A PLATINUM RESISTANT L1210 LINE.

2.1 Introduction.

In a paper published in 1956, Eagle and Foley reported on the cytotoxic action of carcinolytic agents in tissue culture [Eagle and Foley, 1956]. Since then, many tumours have been grown in tissue culture and have played a central role in the screening of novel compounds to assess their potential as anticancer drugs. From the inception of tissue culture, the L1210 murine leukaemia cell line has been the mainstay of drug screening systems, and in more recent years the resistant variants of the L1210 line have become important models against which new drugs can be tested.

The murine L1210 lymphoblastic leukaemia was first induced in mice aged 8 months after repeated skin painting with 0.2% 3-methylcholanthrene in ethyl ether, as first described by Law (Law et al, 1949). Since then, the cell line has been continued by subcutaneous and intramuscular injection in mice. The tumour has been used extensively *in vitro* and *in vivo* because it adapts easily from growth as ascites.

Further analysis of the L1210 line by Dowjat and Wlodarska suggested that L1210 may be a T-cell leukaemia because its karyotype revealed trisomy 15

in 65% of the cells, a feature found in many other murine leukaemias known to be of T-cell origin [Dowjat and Wlodarska, 1981]. Monosomy of the X chromosome was also reported and has previously been seen in transformed lines, human and animal tumours [Sakurai et al, 1974]. There were, however, changes in the cytogenetic characteristics during the course of the L1210 undergoing *in vitro* adaptation.

Like most experimental leukaemias, L1210 is characterised by rapid growth - namely a growth fraction close to 1. This property makes it of great value in the determination of the phase specificity of new cytostatic agents as well as providing a quick screen for effectiveness of novel compounds. Conversely, those who criticise the use of the cell line in screening for selection of novel anticancer drugs maintain that its rapid growth mitigates against the discovery of agents which may be active against the more slowly growing tumours, for example those of the gastrointestinal tract.

Although the trend is now away from the sole use of murine lines and towards human tumour cell culture and xenograft evaluations which may give a more accurate indication of the behaviour of human cancers, the L1210 parent sensitive and resistant cell lines will retain an important position in the screening programme since they provide an essential historical reference to the background of selection of potentially clinically active agents. For example, tetraplatin was chosen to enter clinical trials as

a possible novel platinum anticancer drug on the strength of its activity against an L1210 cell line with acquired resistance to cisplatin [Anderson et al, 1986].

The *in vitro* growth of L1210 and other tumour models is valuable in that resistant lines can be developed, tumour characteristics may be studied and sensitivity to antineoplastic drugs may be established. Investigation into the mechanisms of resistance can then be performed and the cell lines may also be used to screen novel anticancer drugs.

In addition to detailing the method by which platinum resistance was developed in the L1210 cell line *in vitro*, the characteristics of the resistant cell lines have been established. One of the features of a cell which may change with resistance development is the ploidy. It was therefore important to establish whether the L1210 resistant lines had changed from the diploid character of the parent sensitive line using flow cytometry. Since later studies were to require measurement of the cellular protein content, the possibility that resistance development could be associated with a change in cell volume and protein content had to be considered. These parameters were compared in the parent L1210 line and the three variant lines resistant to cisplatin, carboplatin, tetraplatin and iproplatin. The resistant lines are referred to as L1210/cisplatin, L1210/carboplatin, L1210/tetraplatin and L1210/iproplatin. The rate of cell growth, as measured by the doubling time

of the cells, was also established for the resistant cell lines and compared with that of the parent sensitive line.

The sensitivity profiles of the resistant L1210 cells were compared with those of the parent cells using cisplatin, carboplatin, tetraplatin and iproplatin. This allowed identification of patterns of cross-resistance in the variant cell lines and speculation on the importance of structural differences in compounds which can overcome resistance.

Finally, in order to assess the stability of the resistance induced in the L1210 cells *in vitro*, the platinum resistant cells were cultured in drug-free medium for a prolonged period of time with repeat measurement of their degree of resistance at regular intervals.

2.2 Materials and Methods.

2.2a Development of resistance.

All cells were grown in suspension in RPMI 1640 medium supplemented with 10% horse serum, 2mM glutamine and antibiotics (100U/ml penicillin and 0.1mg/ml streptomycin).

Cisplatin, tetraplatin and iproplatin were formulated in 0.9% saline. Carboplatin was dissolved in sterile water.

To produce carboplatin resistance, parent sensitive L1210 cells were exposed continuously to 2.5μM carboplatin. This starting concentration was selected because the IC50 of L1210 to carboplatin was 6μM and it was noted that the sensitive cells had a 70% survival measured by dose/response curve after exposure to 2.5μM carboplatin. Following growth of the surviving fraction, the cells were re-exposed to the same concentration to a total of 8 treatments at 2.5μM. The dose was then escalated to 5μM (9 treatments), 7.5μM (17 treatments), 10μM (18 treatments), 20μM (16 treatments), 30μM (10 treatments) and finally 40μM (23 treatments) over a period of 16 months. At that time the cells were 13-fold resistant to carboplatin when compared with the parent sensitive line, as measured by continuous exposure to carboplatin for 48 hours. Maintenance of resistance was ensured by twice weekly exposure to 10μM carboplatin and the cell line doubling time was tested regularly.

The development of resistance to cisplatin, tetraplatin and iproplatin was done in the same way, with sensitive cells undergoing constant exposure to serially increasing concentrations of each drug. In each case retreatment with fresh drug was instituted when the cells looked healthy and had reached a concentration of at least 5 x 10^4/ml.

The stability of resistance in cells out of regular maintenance doses of cisplatin, carboplatin and tetraplatin was checked by measuring the IC50 at monthly intervals.

2.2b Cytoflourimetry.

i) Preparation of standard.

Two female Balb C mice were sacrificed and their femurs removed. The marrow content was washed out into 4ml PBS (Flow Labs) and brought to a single cell suspension using a 19G needle and a 10ml syringe. The suspension was then spun at 1500rpm for 5 minutes. The supernatant was discarded and the cells were carefully resuspended in 0.2ml ice-cold PBS. A 10μl sample was added to 10ml Isoton and the cell number counted on the coulter counter, model ZM. The remainder of the marrow + PBS had 2ml cold 70% ethanol added and the sample was refrigerated.

ii) L1210 cell preparation.

A total of 5 x 10^6 cells per L1210 cell line were required for cytofluorometric analysis. Each cell line was grown in supplemented RPMI 1640 medium as detailed above to a concentration of 3-4 x 10^5/ml thus ensuring that cells were in mid-log phase at the time of the experiment.

The resistant cells were prepared on the day of the analysis in a manner identical to that outlined for the standards.

2.2c Cell sizing.

The volumes of the sensitive and resistant L1210 cells were measured by coulter counter (model ZM) and pulse height analyser (Mevway) with the help of Dr Trevor Macmillan.

2.2d Measurement of protein content.

The method of Lowry [Lowry, 1951] was used to measure the protein content of the L1210 cell lines, all of which had been grown to a concentration of 4 x 10^5/ml in the supplemented RPMI 1640 medium. In this method, protein is precipitated with alkaline copper treatment and measured using the Folin phenol reagent. A standard curve was prepared using stock protein (Bovine serum albumin) 200μg/ml in 0.1N NaOH. Samples were read in a Beckman spectrophotometer at 750mμ. Results were expressed as μg protein/10^7 cells.

2.2e Measurement of IC50 in L1210 sensitive and platinum resistant cells.

All cell lines were grown to a concentration of 5x10^4/ml in medium as detailed above. Cisplatin was prepared as described previously and added to L1210/s at concentrations 2.5, 1.25, 0.625, 0.3125 and 0.156μM. Cisplatin concentrations added to the L1210/cis cells were 10, 5, 2.5, 1.25 and 0.625μM. In every case the volume of drug aded was 0.1ml to 5 ml cells. Tetraplatin was added to L1210/s cells at concentrations 0.625, 0.3125, 0.156, 0.078 and 0.039μM. Tetraplatin concentrations added to L1210/tetra were 10, 5, 2.5, 1.25 and 0.625μM. Each point was set up in triplicate.

2.3 Results.

Table 2.1 demonstrates the time taken for the development of resistance to the four platinum drugs. The time taken to develop the 10-fold resistant L1210/cisplatin line was 7 months compared with 16 months to develop 13-fold L1210/carboplatin resistance as described above. It took 12 months to develop a 34-fold resistant L1210/tetraplatin line and the new L1210/iproplatin line had 7-fold resistance developed over a period of 18 months.

Table 2.2 summarises the characterisation of the resistant cells as compared with the sensitive parent line. The doubling time of the L1210/carboplatin cells is comparable to that of the parent sensitive line (15 hours v. 13.6 hours). There is no significant difference in protein content or in cell size. All three resistant cell lines remain diploid.

TABLE 2.1

In Vitro Development of Acquired Resistance to Platinum Compounds in the L1210 Cell Line

Compound	Number of treatments	Exposure time	Development time	Fold resistance in suspension culture*
Tetraplatin	38	2 hours	4 months	34
Cisplatin	46	2 hours	6 months	10
Carboplatin	101	continuous	18 months	14
Iproplatin	51	2 hours	18 months	7

***Compared with IC50 value for the sensitive line. The IC50 value was defined as the concentration of compound which reduced cell counts to 50% of control following continuous exposure for 48 hours.**

TABLE 2.2

Biological Characteristics of L1210 Cell Lines

Cell Line	Doubling time (hours)	Cell volume (μ^3)	Protein content ($\mu/10^7$) cells	Pliody
Sensitive	13.6	620	999 (S.E ± 15)	diploid
Cisplatin resistant (10 fold)	14.7	750	1026 (S.E ± 17)	diploid
Tetraplatin resistant (34 fold)	14.2	631	994 (S.E ± 22)	diploid
Carboplatin resistant (13 fold)	15.0	565	1004 (S.E ± 17)	diploid

Figure 2.1 is a series of graphs of the flow cytometry of control, L1210 sensitive and resistant cell lines. The diploid character of the cells is confirmed since all peak between 190 and 240.

Table 2.3 shows patterns of cross resistance to the 'calibrating 'platinum drugs. There is partial cross-resistance to carboplatin (4.8-fold) in the 10-fold L1210/cisplatin line. The 13.7-fold L1210/carboplatin line demonstrates a 10.2-fold cross resistance to cisplatin, but there is no loss of sensitivity to tetraplatin or to iproplatin. The 34-fold L1210/tetraplatin cell line reveals remarkably little cross resistance to cisplatin and carboplatin (3.1- and 3.5-fold respectively) and it remains completely sensitive to iproplatin.

Table 2.4 documents the comparative IC50's of parent L1210 lines against those of the resistant lines which have been grown free of maintenance drug for two years. There is no significant difference in IC50 which confirms that when resistance has developed it is stable even without exposure to the platinum compound to which resistance was developed.

FIGURE 2.1

FLOW CYTOMETRY OF CONTROL MOUSE BONE MARROW SENSITIVE AND RESISTANT L1210 CELLS

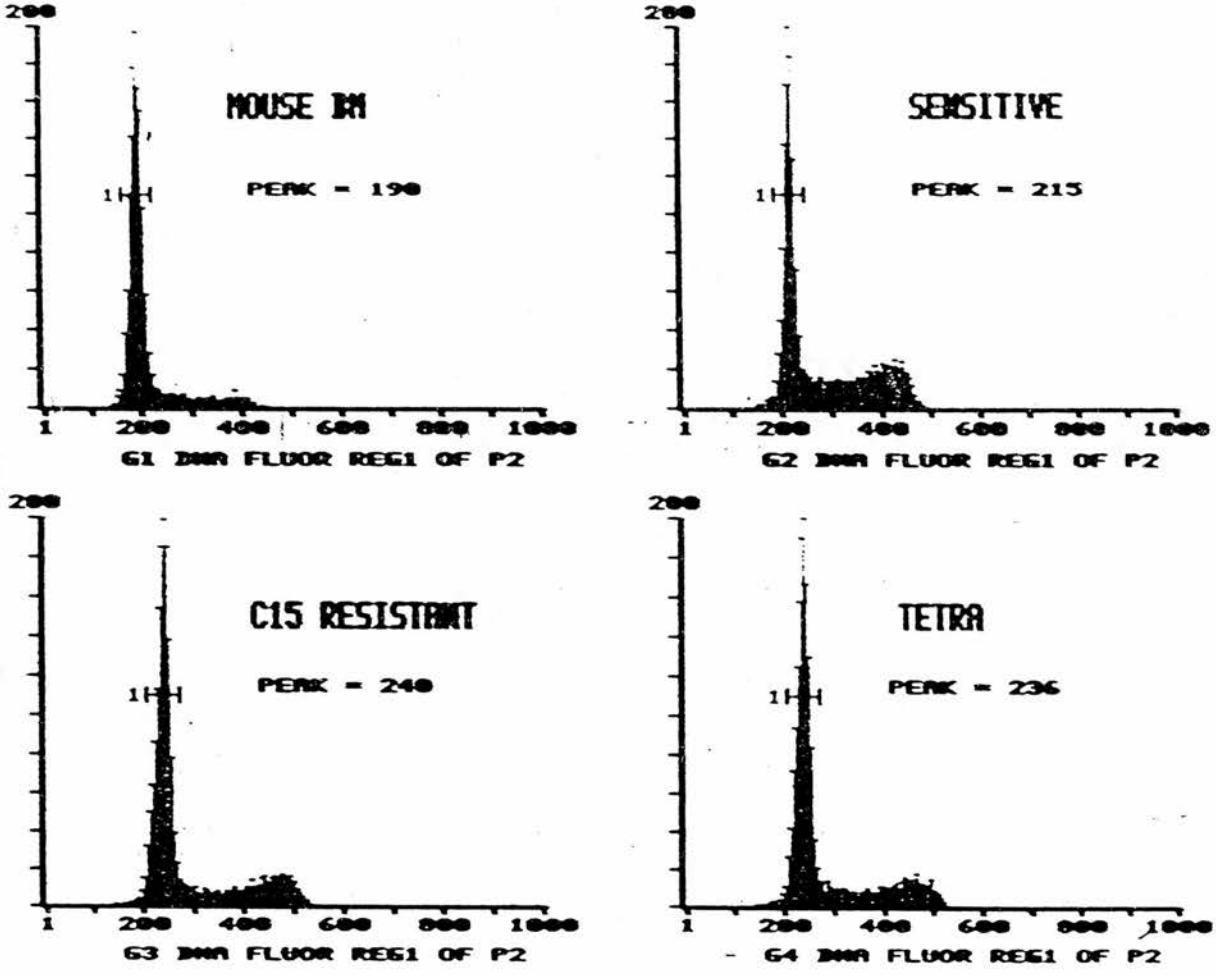


TABLE 2.3

Calibration of variant L1210 cell lines (cell count assay)

	IC50(μM)			
	L1210/S	**L1210/cis**	**L1210/tetra**	**L1210/carbo**
Cisplatin	0.52	5.2 (10)	1.63 (3.1)	5.3 (10.2)
Carboplatin	6.2	29.5 (4.8)	22.0 (3.5)	85 (13.7)
Tetraplatin	0.12	0.14 (1.2)	4.1 (34.0)	0.14 (1.2)
Iproplatin	7.1	9.8 (1.4)	11.4 (1.6)	9.9 (1.4)

TABLE 2.4

Comparative IC50 values for parent L1210 cells and resistant L1210 grown maintenance-free for 2 years.

	IC50 (μM)	Resistance (-fold)
Cell line and drug		
L1210/S v. cisplatin	0.65	-
L1210/cis v.cisplatin	4.2	6.5
L1210/S v.tetraplatin	0.16	-
L1210/tetra v.tetraplatin	4.3	27

2.4 Discussion.

There was a marked difference in the relative ease with which resistance was induced in the three cell lines *in vitro* (Table 2.1). L1210/tetra took only four months and 38 exposures to the tetraplatin to achieve 34-fold resistance compared with the L1210/S. L1210/carbo required continuous exposure to carboplatin for 101 treatments over a period of 18 months before a 14-fold resistance was developed. This finding might suggest that the mechanism for resistance development is different in the L1210 cell line, depending on the structure of the drug to which resistance is induced. The L1210/iproplatin line took eighteen months to reach 7-fold resistance, and the small number of exposures possible in the 18 month period is a reflection of the toxicity of the drug to the cells which had protracted recovery periods from 2 hour incubations in the drug.

There was no significant difference in protein content or in cell size between the parent sensitive and the resistant L1210 cell lines *in vitro* (Table 2.2). All three resistant cell lines remained diploid. These factors were important because in later experiments where platinum uptake and DNA platination are measured, results are expressed in terms of platinum content per mg of protein. A change to tetraploidy would have doubled the amount of DNA and rendered the conclusions invalid. The doubling times of the sensitive and the resistant lines were also very similar (13.6 to 15 hours) and there was no significant in the volume of the resistant L1210 cell lines (620 to

750u[3]). The overall character of the resistant cell lines was therefore very close to that of the parent sensitive line.

Cross-resistance studies were interesting in that there was partial cross resistance to carboplatin (4.8-fold) in the 10-fold resistant L1210/cisplatin line. The 13.7-fold resistant L1210/carboplatin cell line demonstrated a 10.2-fold cross resistance to cisplatin, but there was no loss of sensitivity to tetraplatin or to iproplatin. The lack of cross resistance could be related to the difference in ease of develpoment to the drugs as discussed above. The 34-fold resistant L1210/tetraplatin cell line revealed remarkably little cross resistance to cisplatin and carboplatin (3.1- and 3.5-fold respectively). All cell lines remained completely sensitive to iproplatin.

The development of resistance through exposure of the sensitive cells to increasing concentrations of cisplatin, carboplatin and tetraplatin is a characteristic that remained stable in the cells even when they were cultured in drug free medium for protracted periods of time - up to and beyond two years. This suggested that the mechanism of resistance to the platinum drugs - at least in the L1210 murine leukaemia cells *in vitro* - was a feature retained in the cell memory or genome to enable easy passage to the progeny.

It has not been established whether the resistant cells *in vitro* are representative of the *in vivo* situation, but there are probably several different and specific mechanisms involved to produce the patterns of cross resistance (and lack of it) seen in the L1210 lines discussed above. Later in the thesis, the results of screening of novel platinum based compounds will be presented, and the variability of sensitivity profiles will again give weight to the theory that different types of resistance are involved in the various L1210 sublines.

The usefulness of the L1210 resistant lines in screening of potentially active anticancer agents is only one of their functions. The other important studies facilitated by the development of such lines are those which investigate the means of cells becoming resistant to established antitumour drugs. The following chapter details *in vitro* work on the platinum drugs cisplatin, carboplatin and tetraplatin where the mechanisms for platinum resistance in L1210 cells is explored.

CHAPTER 3

INVESTIGATION OF MECHANISMS OF PLATINUM RESISTANCE IN L1210 CELL LINES

3.1 Introduction.

As discussed earlier, tumours are adept at developing resistance to chemotherapy drugs and a better comprehension of the mechanisms of resistance may allow circumvention of the problem. Platinum resistance may come about through a genetic event which results in any or all of the following:

a) Decreased drug accumulation (alteration in membrane transport)

b) Increased drug extrusion from the cell

c) Activation of cellular thiol mechanisms

- glutathione and other low MW thiols
- metallothioneins

c) DNA related mechanisms

- decreased binding
- increased repair
- increased tolerance of DNA adducts.

In this chapter, alteration in platinum accumulation in the L1210 sensitive and resistant cell lines is measured. In addition to measuring drug uptake,

efflux is assessed since it has previously been established that in some cancer cells resistance to the natural product antineoplastic drugs is mediated by an increase in the ability of the resistant cells to pump drugs out of the intracellular domain, an effect mediated by the p170 glycoprotein located in the cell membrane [Kartner et al, 1983].

Following studies on the accumulation of platinum, colony assay experiments have been used to make an assessment of the ability of the sensitive L1210 and resistant variants to withstand various intracellular concentrations of cisplatin, carboplatin and tetraplatin. A similar experiment comparing the cisplatin sensitivity of Hela (cervical cancer) and V79 (Chinese hamster ovary) cell lines demonstrated that when the survival of the cells in colony assay was plotted against the intracellular platinum concentration per gram of DNA, the more sensitive Hela cells had a lower survival fraction for any given cisplatin concentration [Fraval and Roberts, 1979].

The relative importance in resistance of the other mechanisms outlined, involving thiols and DNA platination, are addressed in chapter 4.

3.2 Materials and methods.

3.2a Growth of L1210 cell lines.

Parent sensitive L1210 cells and their platinum resistant counterparts were

subcultured in RPMI 1640 medium supplemented with 10% horse serum, 2mM glutamine (Flow Laboratories) and antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin).

3.2b Measurement of intracellular platinum concentration.

i)"Uptake".

50ml aliquots of cells in duplicate at 4 x 10^5/ml (mid logarithmic phase of growth) were exposed to varying concentrations of cisplatin or tetraplatin (up to 80μM) for 2 hours or to carboplatin for 24 hours. The longer duration of drug exposure in the case of carboplatin was necessary because of its chemical stability and slower rate of reaction. Cisplatin and tetraplatin were formulated in sterile 0.9% saline. Carboplatin was dissolved in sterile water. Cell suspensions were then centrifuged at 800g for 5 minutes at 4^0C, washed once with phosphate buffered saline (PBS) and the resultant pellets stored at -20^0C prior to analysis. Thawed cell pellets were reconstituted in water (8 x 10^7 cells/ml) and sonicated at 23kHz for one minute. 50μl of sonicate was injected into a Perkin Elmer 306 flameless atomic absorption spectrometer. The temperature was raised initially to 1400^{0}C over 30 seconds and then to 2600^{0}C.

An internal standard curve was constructed from varying concentrations of chloroplatinum in 10% HCl (Aldrich) added to untreated cell sonicates.

ii)"Efflux".

Cells were grown to a concentration of 4 x 10^5/ml prior to being exposed to 40μM drug concentrations for 2 hours (cisplatin and tetraplatin) or 24 hours (carboplatin). Cells were then washed in PBS and resuspended in fresh drug-free medium. Intracellular platinum concentrations were measured following a 2 hour period in drug free medium as described above.

3.2c Measurement of cellular protein content.

Protein content was estimated according to the colorimetric method of Lowry [Lowry, 1951] as detailed in 2.2d. Final results are expressed as nmoles of platinum per mg of protein.

3.2d Assessment of survival of sensitive and resistant L1210 cells in colony assay.

In cell survival assays, 10ml of cells at 2 x 10^5/ml in duplicate were exposed to varying concentrations (up to 80μM) of cisplatin or tetraplatin for 2 hours or carboplatin for 24 hours, followed by centrifugation at 800g, 4^{0}C for 5 minutes. Cell pellets were washed once with phosphate buffered saline (PBS), resuspended in 10ml of complete medium, serially diluted and 2ml aliquots in duplicate added to polypropylene tubes (NUNC) containing 3ml of medium supplemented with 20% horse serum and 0.12% w/v Agar Noble (Difco Laboratories) at 45^{0}C. Tubes were plunged into iced water to set the agar. The tubes were then incubated at 37^{0}C for 7 days after which the

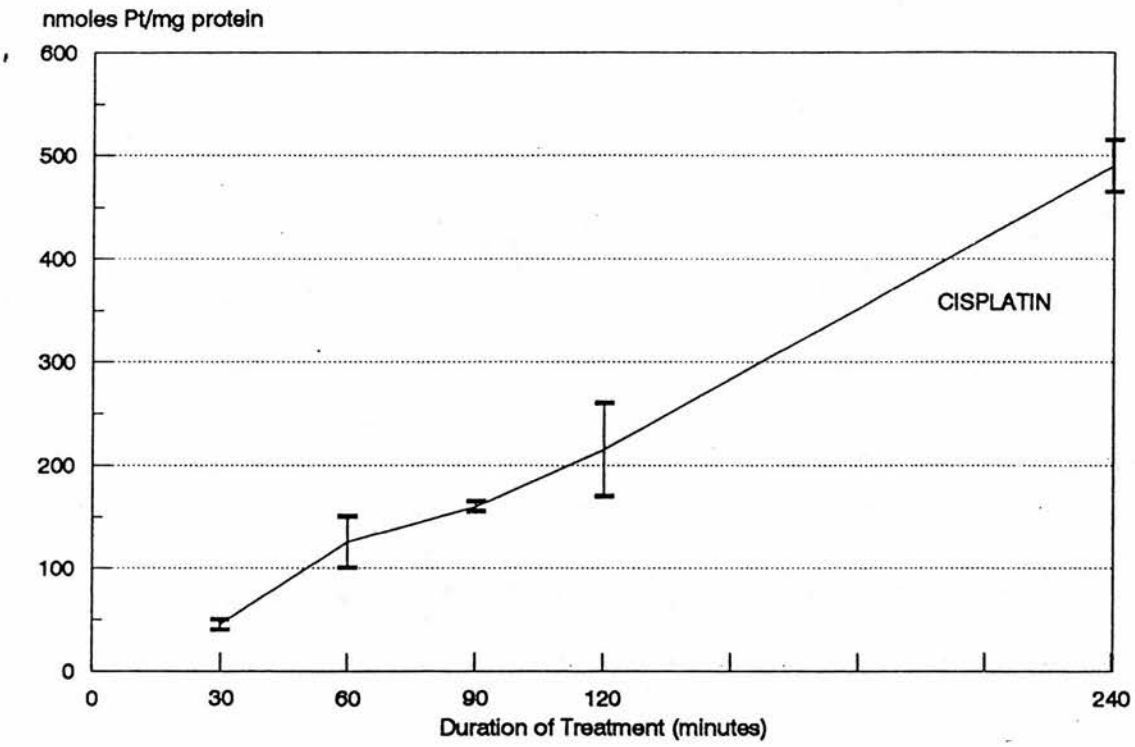
colonies were counted.

3.3 Results.

Figure 3.1 shows the relative amounts of intracellular platinum measured after exposure to 40μM cisplatin for 30, 60, 90, 120 or 240 minutes. To ensure that platinum concentration would be easily measurable in subsequent experiments, it was decided to use the 120 minute exposure time for further uptake experiments involving cisplatin and tetraplatin. At this time point the intracellular platinum concentration after 40μM cisplatin was 200nmoles/mg protein. The cells were exposed to carboplatin for 24 hours because of its slower rate of reaction due to increased chemical stability.

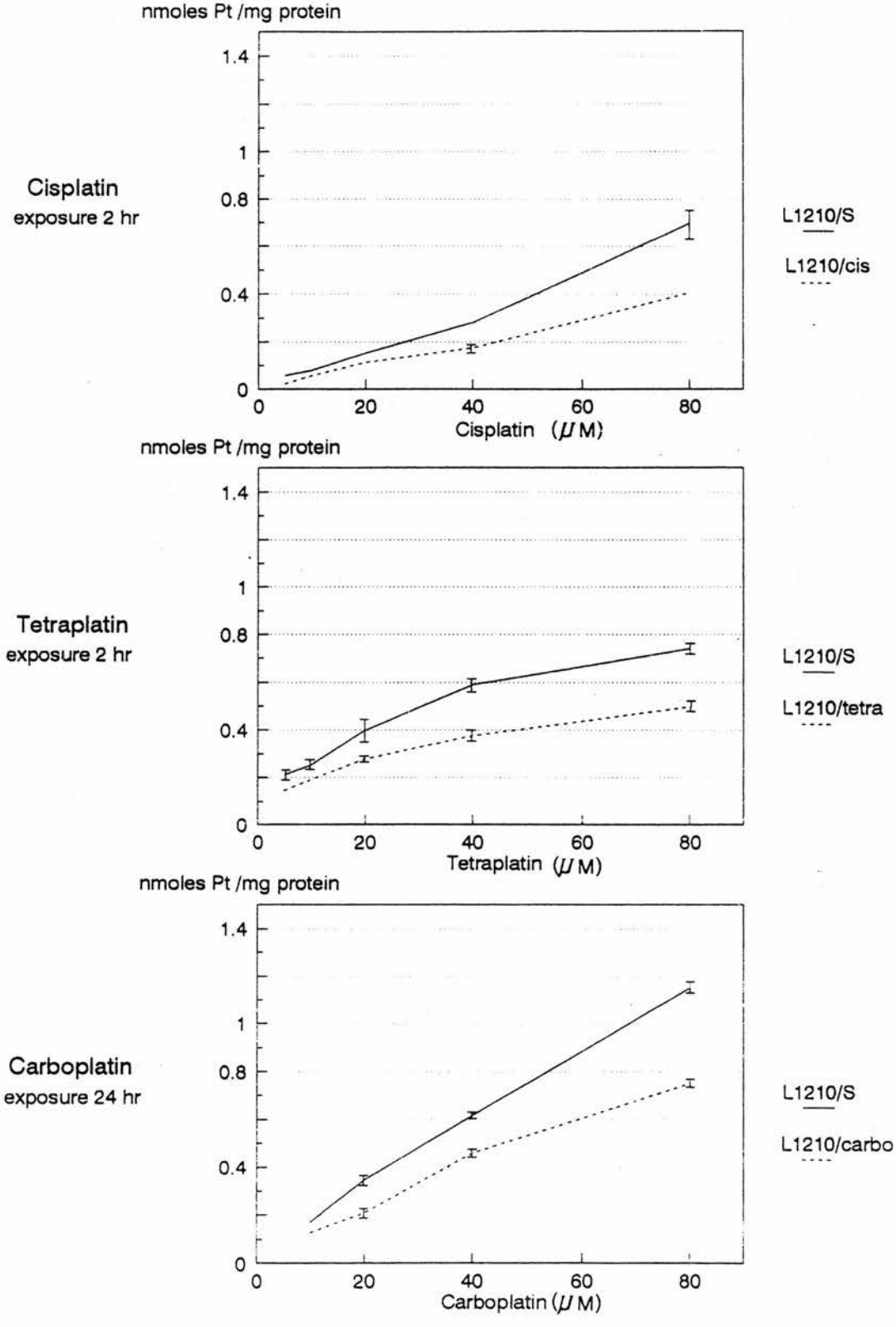
The graphs in Figure 3.2 demonstrate that for all three drugs cisplatin, carboplatin and tetraplatin the L1210 resistant variants contain less platinum than do the parent sensitive (L1210/S) cells. The difference is most significant at the 80μM dose of drug where the reduction in intracellular platinum in the resistant cell lines is 43% (L1210/cis), 33% (L1210/tetra) and 35% (L1210/carbo). The intracellular platinum content of resistant cell lines was comparable after cisplatin and carboplatin at 0.4-0.5nmoles/mg protein, but an increased amount (0.75nmoles/mg protein) was measurable after 24 hours' exposure to carboplatin.

Figure 3.1 nmoles Pt/mg protein in L1210/Cisplatin after exposure to 40μM Cisplatin versus duration of exposure (minutes).



Each point represents the mean of 3 experiments (+/- sd)

Figure 3.2 Intracellular platinum levels in L1210 cells
(Error bars = S.E., duplicate cultures per point)



The rate of loss of platinum from the cells is shown in Figure 3.3. There is no evidence for an increased rate of efflux of platinum from any of the resistant cells when it is compared with efflux from the parent L1210 line. From these graphs the lower uptake of platinum in the resistant cell lines is also evident: After cisplatin exposure the sensitive L1210 have 0.45nmoles of platinum per mg of protein compared with the cisplatin resistant L1210 which contain only 0.2nmolesPt/mg protein.

Figure 3.4 demonstrates the cytotoxicity of platinum drugs in L1210 variants in soft agar colony assays. Plating efficiency of control cultures (400 cells plated) was 37%. Each resistant cell line shows markedly enhanced survival following dosing with equivalent doses of drug. For example, following exposure to 20μM tetraplatin for 2 hours, 0.001% of the L1210/S cells are alive compared with 65% of the L1210/tetra cell line.

The graphs in Figure 3.5 compare cell survival (soft agar colony assay) and initial intracellular platinum concentrations in the L1210 cells. Taking carboplatin as an example, after 24 hours' exposure to 80μM of drug, 0.02% of L1210/S survive with a platinum content of 0.6nmoles/mg protein compared with 50% survival of the L1210/carbo cell line containing the same intracellular platinum concentration. The enhanced ability to tolerate any given concentration of intracellular platinum is seen in all three of the platinum resistant L1210 cell lines.

Figure 3.3 Efflux of platinum from L1210 cells
(Error bars = S.E., duplicate cultures per point)

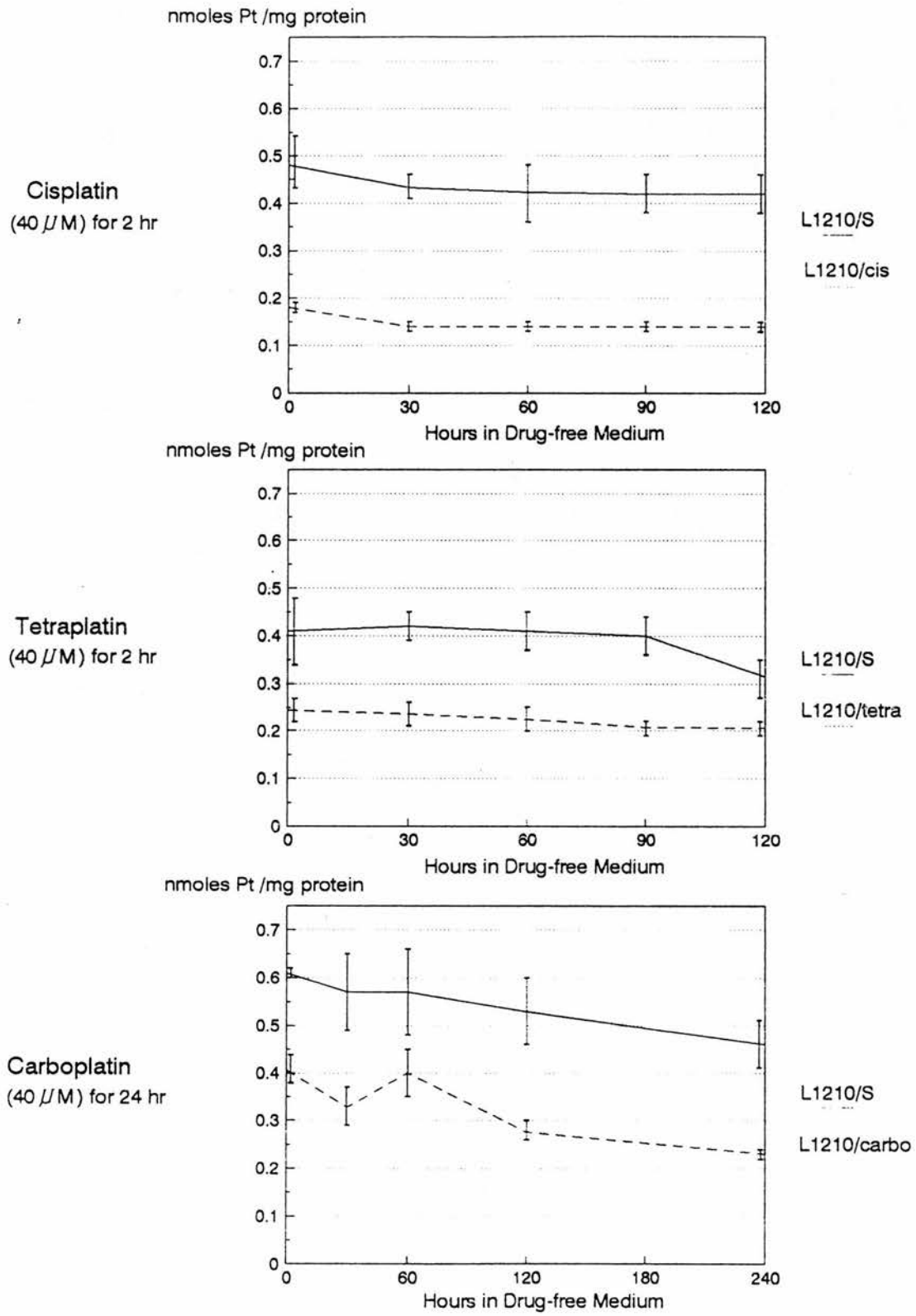


Figure 3.4 Cytotoxicity of platinum to L1210 variants in soft agar colony assays

(each point represents the mean of four separate observations)

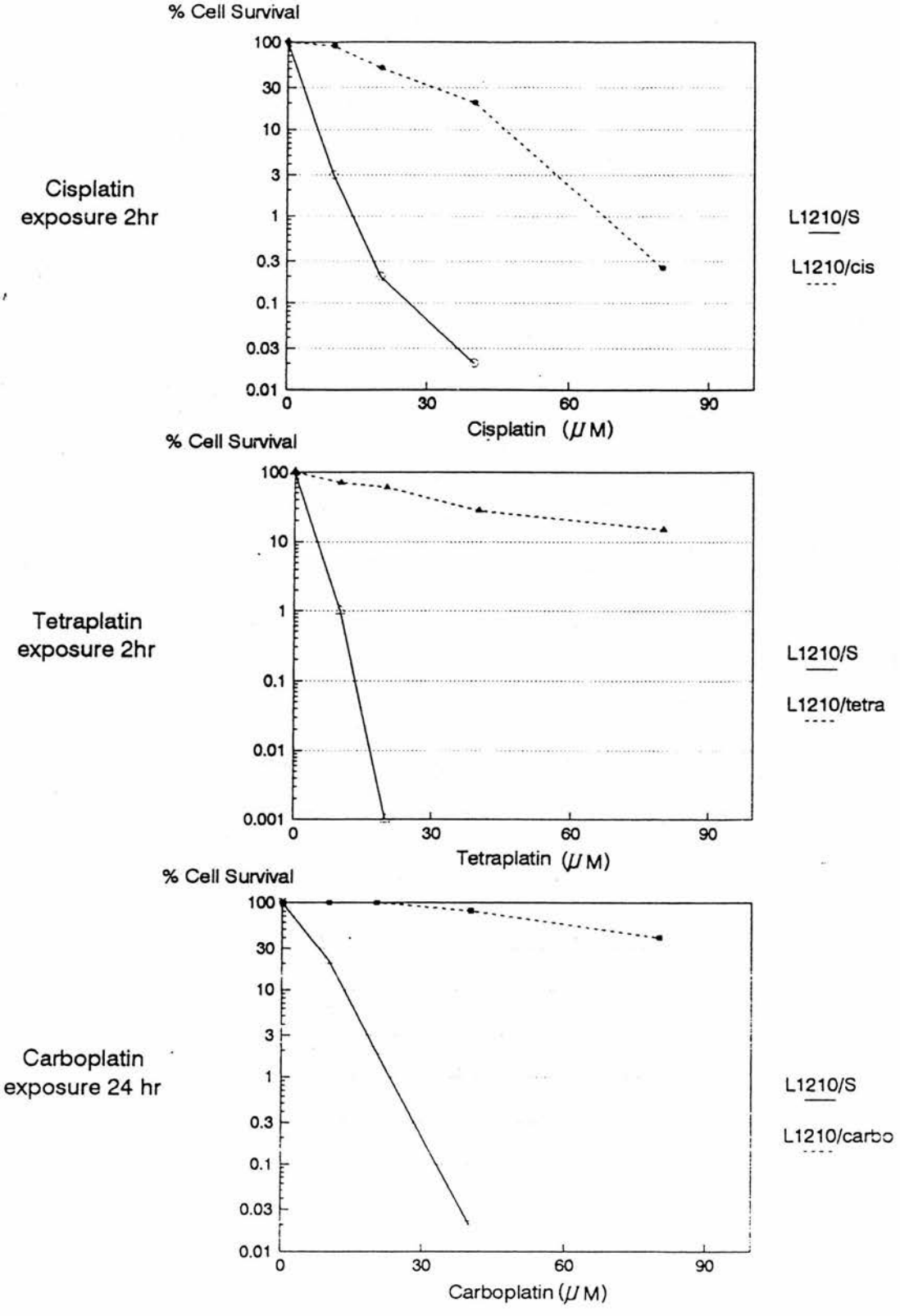
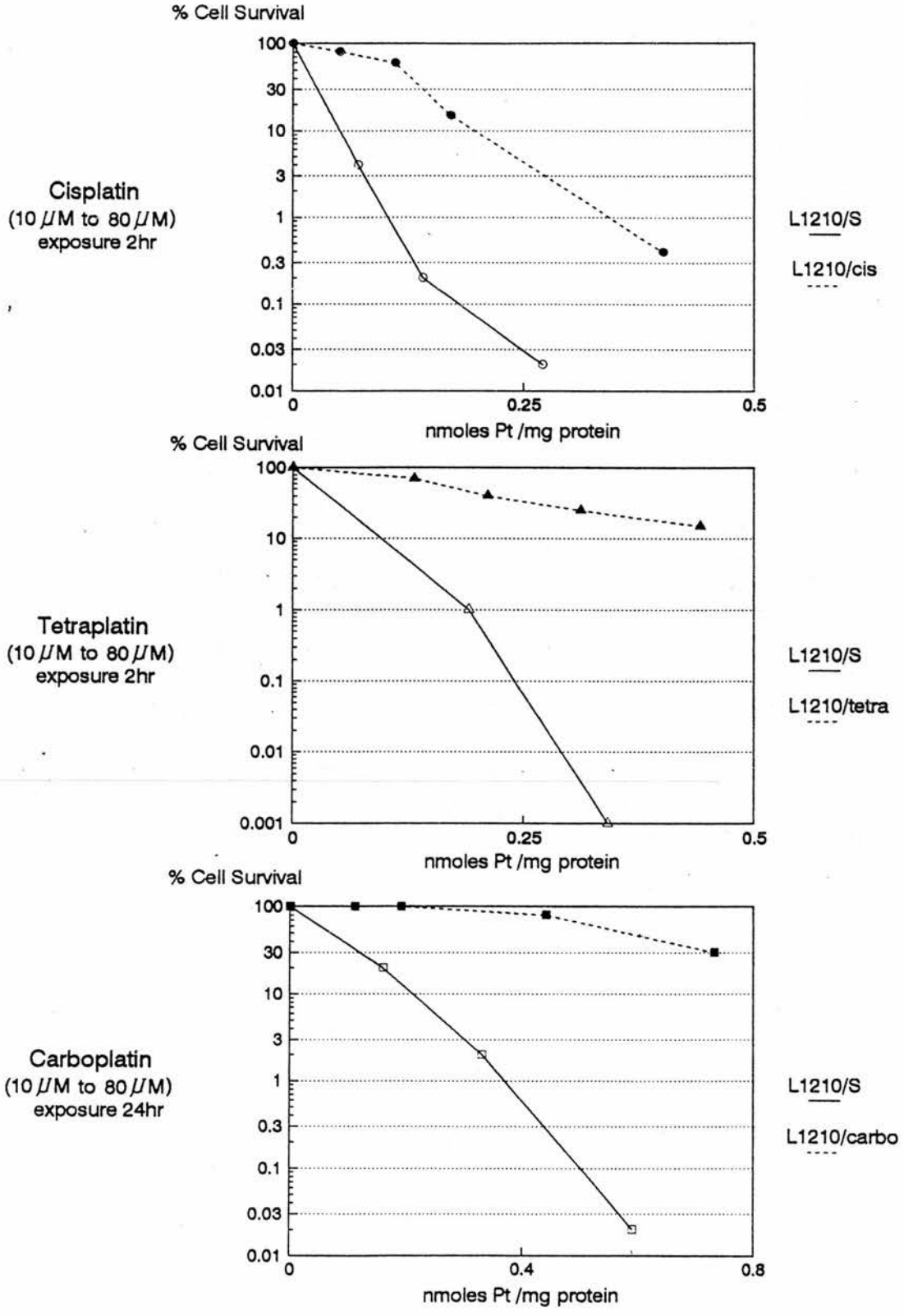


Figure 3.5 Correlation between cell survival (soft agar colony assays) and initial intracellular platinum levels in L1210 cells
(each point represents the mean of four separate observations)



3.4 Discussion.

The results of these experiments involving sensitive and resistant variants of the L1210 cells confirm previous reports that one of the means by which platinum resistance develops in these cells is through a reduction in platinum accumulation.

Before deliberating on the reasons for lower intracellular platinum concentrations found after the resistant cells' exposure to the drug, it is necessary to consider the mechanism by which platinum is taken up into the sensitive cell population. Past investigation into transport mechanisms led to the conclusion that mediated transport of drugs is a xenobiotic phenomenon ie: mediation of entry of artificial substrates occurs by a mechanism already functioning for the accumulation of naturally occurring substrates. These existing mechanisms include passive diffusion across the membrane, active transport (an energy dependent process) and mediated transport which may involve the incoming substance being linked to a carrier of some sort.

Gale and colleagues found that the rate limiting factor for platinum uptake was the external platinum concentration and concluded that cellular entry of cisplatin was by passive diffusion [Gale et al, 1973]. However, Andrews and co-workers argued that since the actual compound on which these experiments were carried out was cis-dichloro(dipyridine)platinum II, it was

possible that the pyridine ligands may have had a significant effect on cellular uptake of the compound, making it poorly predictive for what happens with cisplatin [Andrews et al, 1988]. In the same paper, Andrews stated that the intracellular concentration of cisplatin did not equate with an extracellular concentration of 1μM until 4 hours for the parent sensitive cells and 16 hours for cisplatin resistant cells, thus providing evidence against cisplatin being transported uphill against a concentration gradient by active transport. Likewise, the finding that the intracellular cisplatin aquated and rapidly platinated other cell elements leaving only 10% intact platinum in the cell mitigated against the suggestion that active transport is involved in the process of platinum entering cells, despite the fact that the intracellular platinum concentration eventually surpassed the extracellular concentration - a situation normally indicative of active transport. Conversely, the discovery that Dinitrophenol (an oxidative phosphorylation inhibitor), sodium fluoride and iodoacetate (inhibitors of glycolysis) did decrease accumulation when used in combination led Andrews and his colleagues to conclude that 50% of cisplatin transport was in some way energy dependent.

The evidence against a carrier being involved was the finding that cisplatin uptake was not saturable up to a concentration of 100μM, and that structural analogues of cisplatin did not appear to inhibit competitively cisplatin accumulation [Andrews, 1988].

It has previously been proven that the toxic effect of the highly water soluble cisplatin can be reduced by adding amino acids to the medium [Byfield and Calabro-Jones, 1981a]. Considering this finding together with the documented methionine requirement of sensitive cells, the postulate that cisplatin is taken up into the cell at least partially by an amino acid transport mechanism would appear to be acceptable.

If a carrier dependent transport system for platinum does exist, it follows that resistance to this group of compounds could stem from somatic mutations that yield cells whose membrane carriers are fewer in number or possibly have a decreased drug affinity. In contrast, indications for the involvement of a carrier system in cellular platinum uptake was provided by Gross and Scanlon who demonstrated altered membrane transport properties in L1210 sensitive compared with cisplatin resistant cells [Gross and Scanlon, 1986]. The sensitive line exhibited a greater dependence on exogenous methionine for optimal cell growth than did the line resistant to cisplatin. There was a 2- to 5-fold decrease in initial uptake of methionine, methylaminoisobutyric acid and threonine in the resistant cells. Altered substrate specificity for methionine and threonine in L1210/cisplatin was demonstrated by amino acid competition experiments. These findings for L1210 also held true for the situation in K562, a chronic myelogenous leukaemia cell line, and for L562/cisplatin cells where changes in plasma membrane essential amino acid transport systems were concomitant both

to the development of resistance and to the restoration of sensitivity to cisplatin.

Cisplatin is known to bind to cell membranes and may cause membrane lesions. Gale and colleagues stated that heavy metals and compounds which compromise membrane permeability enhance the amount of platinum bound per cell [Gale et al, 1973]. An increased ability of the cell to prevent or repair membrane lesions may be one method of developing resistance. This can not be the whole story, however, since Hromas demonstrated that reduced accumulation is not simply a membrane-mediated phenomenon by preincubating resistant cells with drugs which increase membrane permeability to prove that their sensitivity to anticancer drugs was not restored [Hromas et al, 1987]. In a similar study, Mann compared the lipid content, surface membrane fluidity and effect of temperature on cisplatin accumulation in sensitive and resistant human ovarian cancer cells, and discovered no obvious difference in the characteristics between the two cell lines [Mann et al, 1988]. In earlier experiments, Richon had demonstrated that increasing the degree of resistance in cells did not produce a corresponding decrease in their accumulation of drug (Richon et al, 1987). These findings mitigate against a straightforward membrane change being responsible for resistance development.

A decrease in the ratio of sodium potassium adenosine triphosphate (Na^+,K^+- ATP) molecules to protein was found in a human ovarian carcinoma cisplatin-resistant cell line (2008/DDP) which accumulated less cislatin due partially to alteration in the potential of plasma and mitochondrial membranes [Andrews et al, 1991]. It was concluded that cisplatin uptake was partially Na^+ dependent and that maintenance of the Na^+ pump by Na^+,K^+-ATPase would play an important role in determining how much cisplatin entered the cell.

Experiments detailed in this chapter do not suggest an increase in platinum efflux as a mechanism for cellular platinum resistance in the L1210 cell line, making it very unlikely that the multidrug resistance gene (mdr1) is relevant in these cells.

The second set of experiments described in this chapter demonstrate the increased survival in colony assay of resistant L1210 cells over sensitive cells for any given concentration of intracellular platinum. This finding supports the theory that there are factors other than reduced accumulation involved in the resistance of tumour cells to the platinum anticancer drugs.

Although the mechanism behind improved survival of platinated DNA in resistant cells is obscure, the possible explanations include:

1) resistant cells have less platinum attached to their DNA,

2) the DNA sites platinated in resistant cells are different from, and less

important than, those platinated in sensitive cells, or

3) the resistant cells are better able to recognise and repair the DNA damage induced by the platinum.

Experiments which help to determine the answers to these questions are described in the next chapter.

CHAPTER 4

INVESTIGATION OF THE ROLE OF GLUTATHIONE AND DNA PLATINATION IN RESISTANCE

4.1 Introduction.

Glutathione (GSH) is a tripeptide which is the major intracellular non protein sulfhydryl compound. Its functions are protean and include detoxification of xenobiotics, amino acid transport, synthesis of DNA precursors and protection from free radical damage. As early as 1963 it was stated that the ratio of protein-free to protein-bound sulfhydryl compounds correlates with the therapeutic response of selected tumours [Calcutt and Connors, 1963]. Measurement of the levels of glutathione in sensitive and resistant cell lines confirms that in some cells an elevated concentration of GSH is associated with increased resistance to anticancer drugs [Arrick and Nathan, 1984; Russo et al, 1986].

It has previously been reported that in several cell lines the sensitivity to anticancer drugs can be increased by chemically mediated reduction of the intracellular glutathione using the specific γ-glutamylcysteine synthetase inhibitor buthionine sulfoximine (BSO) [Hamilton et al, 1985; Hromas et al, 1987]. In other studies there was no effect on cisplatin cytotoxicity following BSO-induced GSH reduction [Richon et al, 1987; Teicher et al, 1987].

To date there has been no firm conclusion regarding the mechanism of the increased resistance to platinum drugs associated with GSH elevation, but several theories have been submitted:

a) Alteration of platinum membrane transport.

b) Drug inactivation by the formation of an inactive GSH-platinum complex in the cytosol.

c) Decreased binding of the platinum to DNA.

d) Increased repair of the DNA lesions [Behrens et al, 1987]. The mechanisms are discussed in detail later (section 4.4).

In this chapter, the results are presented of GSH levels measured in the parent sensitive L1210 compared with the levels in the resistant lines L1210/cisplatin, L1210/tetraplatin and L1210/carboplatin. The possibility has been explored that the GSH concentration in the cells may change after platinum exposure. The effect on sensitivity of decreasing the intracellular GSH has been investigated by exposing the resistant cells to BSO and subsequently measuring their IC50 against cisplatin, carboplatin or tetraplatin.

Finally, to assess whether decreased binding of platinum to DNA does play a role in platinum resistance in the L1210 cell line *in vitro*, the platinated DNA has been extracted and measured by atomic absorption spectrophotometry.

4.2 Materials and Methods.

4.2a Measurement of GSH.

All L1210 cell lines were grown in supplemented RPMI 1640 medium as detailed in section 2.2a.

Cells were diluted to 2.5×10^4/ml with fresh medium on the day prior to each experiment to normalise for extracellular cysteine content [Post et al, 1983].

GSH levels were measured in the following experiments:

i) in untreated sensitive and resistant cell lines over a 48 hour culture period,

ii) in sensitive and resistant cells which had been exposed to 40μM cisplatin or tetraplatin for 2 hours, or to 40μM carboplatin for 24 hours (the concentration used for platinum accumulation studies), and

iii) in sensitive and resistant cells exposed to the relevant IC50 concentrations (cell count assay, 48 hours' continuous exposure) for each compound.

At each time point 10^7 cells, in duplicate, were harvested by centrifugation and washed twice with phosphate buffered saline (PBS). Cell pellets were stored at -40^{0}C prior to analysis and then lysed in 1ml of distilled water and deproteinised by addition of sulphosalicylic acid (to 3% final concentration). Samples were centrifuged at 10,000g for one minute in a Beckman microfuge and the supernatant assayed for total glutathione as described by

Suzukake [Suzukake, 1982] using the enzyme recycling method of Griffith [Griffith, 1980].

The incubation mixture for determination of total GSH consisted of 100μl of 1M triethanolamine HCl buffer (pH 8.0), 700μl of 0.3mM NADPH, 100μl of 6mM DTNB and 0.48 units of glutathione reductase. All reagents were prepared in 125mM sodium phosphate buffer containing 6.3mM sodium EDTA (pH 7.5). The absorbance of 2-nitro-5-thiobenzoic acid at 412 nm was monitored spectrophotometrically.

Results were expressed as μmoles of glutathione per mg of protein. Reduced glutathione was then derivitised with 2-vinylpyridine but no oxidised glutathione could be detected in the above samples.

In glutathione depletion studies, buthionine sulphoximine was added to cell cultures ($2x10^4$/ml) to a final concentration of 25μM (a concentration which was non-cytotoxic to cells over a 72 hour incubation period) 24 hours prior to addition of platinum compounds. IC50 concentrations after a further 48 hours and glutathione levels at 4, 24 and 48 hours were assessed as described above.

4.2b DNA extraction experiments.

One litre of each cell line at 2 x 10^5 cells/ml (2 x 10^8 cells total) were required for each drug concentration. The cells were exposed to cisplatin at 10, 20. 40 and 80μM for 2 hours. They were then centrifuged for 10

minutes at 3500g, 4^{0}C in a Beckman J6-B centrifuge with zero braking. The supernatant was discarded and the cells were washed once in ice-cold PBS by resuspension and centrifugation. The cells were then centrifuged as above, the supernatant decanted and the pellets were flash-frozen and stored at -20^{0}C until further analysis.

The pellets were thawed into lysing solution (6% w/v paraaminosalicylic acid, 1% w/v sodium chloride, 1% w/v triisopropylnaphthalene sulphonate and 6% v/v secondary butanol in aqueous solution) at a concentration of 1ml lysing solution per 2 x 10^7 cells. An equal volume of phenol reagent (500g phenol, 0.5g 8-hydroxyquinoline, 75ml of m-cresol and 55ml of water) was added and the tubes mixed by inversion to partition out the protein. The solution was transferred to polypropylene tubes and centrifuged at 4000rpm, 10^{0}C for 10 minutes. The upper aqueous layer was removed into 50ml glass conical flasks with stoppers. Double the existing volume of ethoxyethanol was added (to precipitate the DNA) and the flasks were then gently agitated on a shaker for 5 minutes. The DNA was removed with a spatula and 30ml ethanol was added to rinse away the solvents. The DNA was washed a further twice with ethanol and the ethanol was then drained off. The DNA was redissolved in 4ml deionised water over a period of 1 hour. Sodium acetate 10% was then added to bring the final sodium acetate concentration to 1% in order that the DNA be brought down as a sodium salt. 100μl of 1% RNAase was then added and the tubes placed in a water bath for 10 minutes. 100μl proteinase K was

then added and the solution incubated for a further 30 minutes at 37^{0}C. After transferring the contents to a polypropylene tube, an equal volume of phenol reagent was added and the tubes were shaken well. Tubes were then centrifuged at 4000rpm at 10^{0}C for 5 minutes. The upper layer was removed into universal containers and ethanol was again added to reprecipitate the DNA. The DNA was washed twice more with ethanol and finally with ether and transferred to preweighed, labelled microfuge tubes. The ether was drained off and the tubes were left in a 37^{0}C incubator to dry. The tubes were finally placed in a dessicator under vacuum for a 48 hour period to remove any water still associated with the DNA.

4.2c Measurement of DNA platination.

Cells at a density of 4 x 10^5/ml (5 x 10^7 cells per point) were exposed to various concentrations of cisplatin (up to 80μM) for 2 hours. Cell pellets were washed with PBS and incubated overnight at 37^{0}C in lysing solution (10mM TRIS, 10mM edta, 0.15M NaCl, 0.4% SDS) containing 1mg/ml proteinase K. Residual protein was removed by extraction with phenol reagent (500g phenol, 75ml cresol, 55ml water and 0.5g hydroxyquinolone). Following centrifugation, DNA was precipitated from the aqueous phase with ethanol, washed twice with 80% ethanol, dried and dissolved in TE buffer. After incubation with 1% ribonuclease to remove contaminating RNA, the solutions were re-extracted with phenol reagent, DNA was reprecipitated as described above and digested in 0.2% nitric acid prior to

analysis. Platinum associated with DNA was measured by flameless atomic absorption spectroscopy on a Perkin-Elmer 1100b AAS.

Calibration of the AAS was achieved through injection of 50μl samples of 12.5, 25, 50, 75 and 100ng chloroplatinum prior to and subsequent to measurement of the experimental samples.

UV absorbance of diluted DNA hydrosylate (5μl + 4ml water) was used to measure the amount of DNA present. Results were calculated from the knowledge that 1mg/ml DNA gives an optical density of 26 at wavelength 266.

4.3 Results.

Table 4.1 lists the glutathione levels as measured in L1210 sensitive cells compared with L1210/cisplatin, L1210/carboplatin and L1210/tetraplatin from time zero prior to exposure to any drug to 48 hours after passage. It can be seen that there is little difference in the GSH concentrations between sensitive and resistant cells. In agreement with Batist and colleagues [Batist et al, 1986b], cellular GSH levels were dependent upon time after subculture in fresh medium and were lower at 48 hours compared with earlier time points. While the GSH content of the L1210/cisplatin and L1210/tetraplatin were comparable with that of the L1210/sensitive line, the GSH content of the L1210/carboplatin was approximately twice that of the parent sensitive line during the first 24 hours of culture.

TABLE 4.1

Serial determinations of glutathione levels in L1210 variants during cell culture

Time after Passage (hours)§	Total Glutathione Content (mmols/mg protein)			
	L1210/S	L1210/cis	L1210/tetra	L1210/carbo
0	78 ± 20*	91	60	147
4	58 ± 12	76	83	119
24	64 ± 14	61	55	130
48	45 ± 18	49	47	66

* Each value represents the mean of determintions from duplicate cultures unless otherwise indicated.

§ Starting cell densities range from 3.1 to 6.8 $x10^4$ cells/ml

Table 4.2 demonstrates the GSH levels measured at 4, 24 and 48 hours in resistant cells exposed continuously to the IC50 or exposed for 2 hours to 40μM concentrations of cisplatin or carboplatin. With carboplatin, the cells were exposed to the IC50 (6.2μM) or 85μM continuously or to 40μM for 24 hours. Neither tetraplatin nor carboplatin exposure resulted in alteration of the cellular GSH content of sensitive and resistant L1210 cell lines. There was a 36% increase in GSH content of the L1210/cisplatin 24 hours following exposure to 40μM cisplatin for 2 hours but the change was not sustained at 48 hours.

Table 4.3 shows that 24 hours after treatment with BSO 25μM there is a reduction of the GSH content by >75% in all the resistant cell lines. The GSH remained depleted by >50% after 72 hours' continuous exposure. The final column shows the ratio of the IC50 values in the three resistant cell lines expressed as a percentage of the IC50 without BSO exposure. The L1210/carboplatin cell line demonstrated a fall in IC50 indicative of increased sensitivity to platinum (ratio of IC50's 79%) but there was no return of sensitivity to L1210/cis and L1210/tetra.

TABLE 4.2

Total glutathione content (mmols/mg of protein) of variant L1210 cell lines exposed to cisplatin, tetraplatin or carboplatin

Hours Post Drug Addition	Cisplatin				Tetraplatin				Carboplatin		
	Continuous		2 hours		Continuous		2 Hours		Continuous		24 Hours
	0.52μM L1210 /S	5.2μM L1210 /cis	40μM L121 0/S	40μM L1210 /cis	0.1μ/M L1210 /S	40μM L1210 /tetra	40μM L1210 /tetra	40μM L1210 /tetra	6.2μM L1210 /S	85μM L1210 /carbo	40μM L1210 /S
4	56	87	65	91	46	63	52	59	82	199	78
24	56	69	82	124	52	68	62	60	90	172	97
48	46	36	ND	68	24	52	ND	29	75	110	94

Each value represents the mean of determinations from duplicate cultures at the indicated times.

ND = Not determined

Starting cell densities ranged from 3.2 to 6.8 x 10^4 cells/ml.

TABLE 4.3

3
Reduction in glutathione content of platinum resistant L1210 lines following exposure to buthionine sulphoximine (25μM) and comparative cytoxicity studies.

	Total Glutathione (% Reduction)			IC50 values**
	24hr*	48 hr*	72hr*	% + BSO / - BSO
L1210/cis	76	65	60	94[(a)]
L1210/tetra	81	76	51	116[(a)]
L1210/carbo	78	83	72	79 [(c)]

* Hours post BSO addition (continuous exposure)

** Cells were exposed to either (a) cisplatin, (b) tetraplatin or (c) carboplatin, 24 hours after BSO addition and incubated for a further 48 hours. IC50 values were compared with those obtained without BSO. Results are the means of duplicate determinations.

Figure 4.1 demonstrates the decrease in the amount of platinum bound to the DNA of the resistant cells (L1210/cisplatin or R on graph) following exposure of the cells to 10, 25, 50 or 100μM cisplatin for 2 hours. The difference is most marked at the 80μM cisplatin concentration where average DNA platination is approximately 50% less in the resistant cell line (R) than in the parent sensitive L1210 (S).

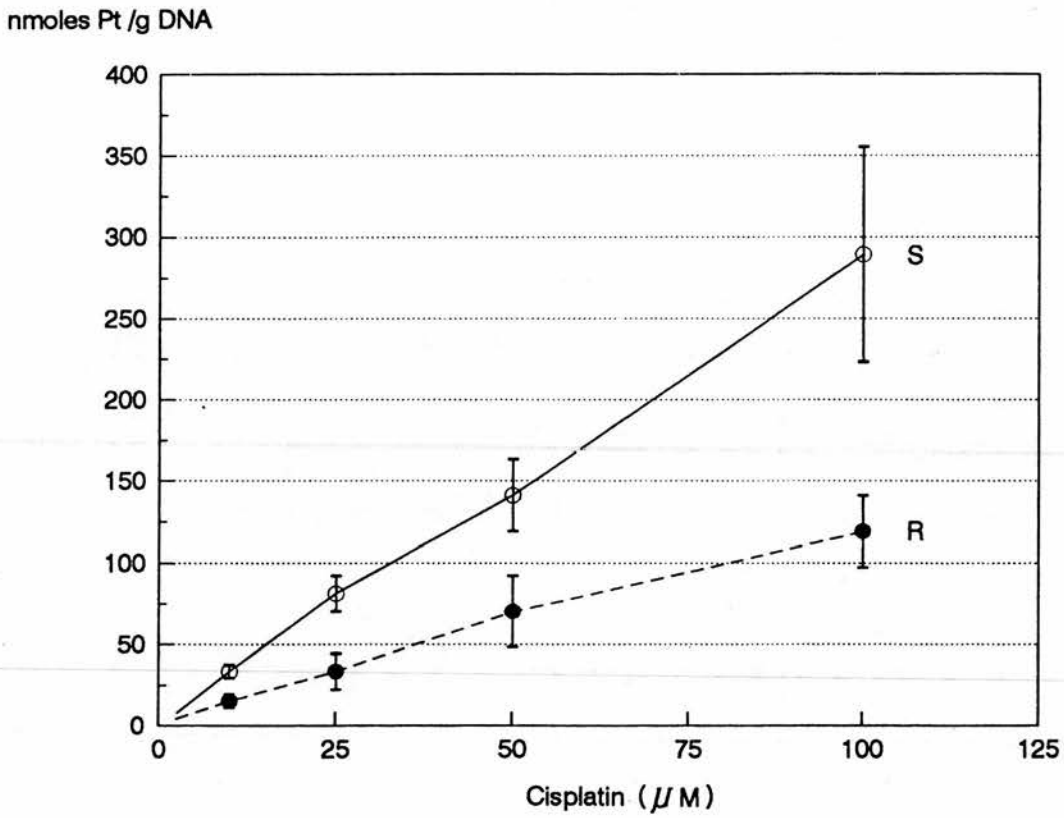
4.4 Discussion.

These experiments demonstrate that at the time points examined, there is no significant elevation in glutathione in our platinum resistant L1210 cell lines. Other groups have also demonstrated that GSH appears to be unrelated to L1210 platinum resistance [Andrews et al, 1987; Richon et al, 1987; Waud, 1987].

Depletion of the intracellular GSH by incubation of the resistant L1210 cells with BSO had remarkably little effect on their sensitivity to the platinum compounds. Following BSO exposure, cellular GSH concentrations were reduced to 24% of the untreated concentrations and alteration in sensitivity should have been seen if GSH were an important factor in platinum resistance in these cells.

In many human ovarian cell lines grown *in vitro*, GSH concentration is elevated in drug resistant variants. In the 2780 ovarian cancer cell line,

Figure 4.1 Pt/DNA Binding in L1210 Cell Lines
after 2 hours (error bars = s.d.)



which was cloned from an untreated patient, the GSH level in the cisplatin variant line was three times higher than that found in the parent sensitive strain [Louie et al, 1985]. The OVCAR3 and OVCAR4 ovarian cancer cell lines were grown from patients who had already developed resistance to radiation, to cisplatin and to other antineoplastic drugs. They also had very high GSH concentrations. In contrast, the COLO316 and 2008 human ovarian cancer cell lines (in which Andrews and colleagues developed cisplatin resistance) showed no elevation of their GSH levels as resistance appeared [Andrews et al, 1985].

At the Institute of Cancer Research, a relationship between intracellular GSH levels and platinum cytotoxicity has been established for eight human ovarian carcinoma cell lines which demonstrate varying sensitivities to the four platinum drugs cisplatin, carboplatin, tetraplatin and iproplatin [Mistry et al, 1991]. The intracellular GSH concentration was related to the cells' resistance to cisplatin, carboplatin and iproplatin, but not to the tetraplatin resistance. Following depletion of GSH with BSO, there was a differential effect on the sensitivity to the platinum II compounds (cisplatin and carboplatin) compared with the platinum IV compounds (tetraplatin and iproplatin) in that the cytotoxicity of the latter drugs was substantially enhanced in all cell lines. There was enhanced cytotoxicity of cisplatin and carboplatin only in one relatively sensitive and in one of the two relatively resistant cell lines.

In BSO depletion experiments with cisplatin resistant 2008 ovarian cells, it was found that prolonged incubation with BSO was necessary to obtain a reduction in GSH significant enough to enhance cisplatin cytotoxicity [Andrews et al, 1988]. Poor recovery and increased cisplatin cytotoxicity were also to be found in resistant cells maintained in stationary phase for 4 - 96 hours prior to placing cells in conditions that permitted cell division. The oxidative stress of passaging A2780 cells can induce an increase in the GSH peroxidase and reductase which peaks on day 2 in the resistant cells, compared with a peak on day 4 seen in sensitive cells [Batist et al, 1986]. Hamilton and colleagues found no evidence of increase in any of the GSH related enzymes in the A2780 cell line, but the enzymes were measured at only one time point in untreated cells [Hamilton et al, 1985].

One of the other postulates at the start of this chapter was that elevation of intracellular GSH may result in a decrease of the platinum binding to the DNA. Work by Andrews and colleagues has shown that GSH detoxifies intracellular platinum and protects DNA from platinum-induced damage [Andrews PA 1986]. To recapitulate on the evidence behind the central role of DNA in platinum toxicity, Roberts showed in 1981 in the ADJ/PC6 tumour *in vitro* that, taking account of the molecular weights, DNA was more extensively platinated on a molecular basis than either RNA or protein [Roberts, 1983]. The crosslinking of DNA - as described in section 1.1 - inactivates it as a template for further replication.

In the experiments investigating DNA platination described above, it was demonstrated that there was reduced platination of the DNA in the resistant L1210 cells (Figure 4.1). Since it has already been shown that there is no elevation of GSH in these resistant cells, it is not possible to challenge the statement that in the L1210 cell line *in vitro*, GSH does not protect a critical DNA target [Andrews et al, 1986], but the findings do contrast with the results of Foka who stated that cisplatin resistant and sensitive L1210 have a similar amount of a cisplatin dose (about 20%) bound to the DNA [Foka et al, 1988].

Others have also shown a relationship between DNA platinum adducts and tumour sensitivity. Reed and co-workers found that in patients with ovarian cancer, sensitivity to single agent cisplatin or carboplatin was associated with an increased number of DNA platinum adducts on their leucocytes than were found in the patients who had resistant disease [Reed et al, 1987]. In that study, a cisplatin-DNA ELISA was used to measure adduct formation. The correlation between better tumour response to platinum and an increased number of cisplatin-DNA cross-links in leucocytes was also reported Fichtinger-Schepman and colleagues [Fichtinger-Schepman et al, 1987].

In addition to measuring the number of platinum-DNA adducts, it is also necessary to consider the importance of the ability of the cells to repair the

damage to their DNA because a difference may exist in the capacity for repair between sensitive and resistant cells. The rate of repair of platinated DNA has not been studied in our L1210 cells, but a discussion of the available information follows.

It has been suggested that resistant lines are better able to survive platinum adducts on their DNA because they exhibit more efficient recognition and repair of the bidentate interstrand X-link between adjacent N^7 guanines [Strandberg et al, 1982; Behrens et al, 1987; Masuda et al, 1988]. Some cells which are less effective in removing interstrand cross links manifest increased sensitivity to cisplatin - for example, repair-deficient Chinese hamster ovary cells [Meyn et al, 1982] and cells derived from people who suffer from Fanconi's anaemia [Plooy et al, 1985]. Investigation of a human ovarian cancer cell line (A2780) which was 5-fold resistant to cisplatin showed that it demonstrated a 2-fold increase in the ability to repair cisplatin-induced damage compared with the parent sensitive line. It has also been noted that after treatment with the specific DNA polymerase-a inhibitor Aphidocolin, there was dose-dependent inhibition of recovery of damage post cisplatin in the resistant cells and an increase of cisplatin toxicity in these cells [Masuda et al, 1988; Lai et al, 1988].

Alternatively, it might be supposed that a cell line which demonstrated an enhanced ability to repair DNA cross-links induced by platinum would be

able to replicate almost normally. In an elegant series of experiments where a resistant human bladder cancer cell line (RT112) and a platinum sensitive germ cell tumour cell line (SUSA) were compared for rate of repair of cross-links, it was demonstrated that SUSA cells were unable to repair DNA-DNA interstrand cross-links, whereas 50%-85% of these lesions were removed in RT112, 24 hours after exposure to cisplatin [Bedford et al, 1988]. Moreover, the interstrand cross-links continued to form over the entire 24 hour period of observation in the SUSA, while the repair component became dominant over interstrand cross-link formation in the RT112 at 14 hours.

A further explanation of resistance, suggested at the beginning of the chapter, was that a population of resistant cells may be less sensitive to the DNA cross-links induced by platinum. Hansson and colleagues found that some human melanoma cells have a higher capacity to maintain DNA synthesis in the presence of DNA cross-links [Hansson et al, 1988]. It has not been established whether this is because of a lower sensitivity of the DNA replicating enzymes of these cells to the presence of DNA interstrand cross-links.

To return to the L1210 cell lines used to generate results discussed in this chapter, it has been shown that in cisplatin resistant L1210 there was an increased capacity for repair of DNA but the extent of repair did not correlate directly with the degree of resistance of the cells [Eastman and Schulte, 1988].

Further investigation into the mechanisms of platinum resistance in L1210 cells is under way. From the experiments described above, it is clear that there is little evidence to support any single theory as an explanation for resistance.

Moreover, whatever light is eventually shed on the mechanisms of development of platinum resistance in cancer cells - and even if some means to reverse resistance is identified - it will remain important to discover and develop new drugs which will be active against resistant tumours. The background to the search for novel antitumour drugs and an appraisal of some new compounds will be presented in the following chapters.

CHAPTER 5

IN VITRO SCREENING OF NOVEL PLATINUM COMPOUNDS FOR CLINICAL USE

5.1 Introduction.

The background to the development and current use of the L1210 murine leukaemia in the screening of novel compounds has been discussed in Chapter 2. The process of early assessment of potential anticancer drugs will be addressed in this chapter.

The advantages of using an *in vitro* system to study the potency of novel compounds include

i) facilitation of investigation of the biology and chemistry of cancer,

ii) provision of information on the molecular basis of cytotoxicity and

iii) reduced cost compared with *in vivo* work.

Whereas the L1210 and P388 cell lines have advantages in tissue culture because of their rapid growth and ease of manipulation, human tumours established in culture provide improved clinical predictiveness especially where tumour xenografts can be developed. The rationale is that human cancer cells in vitro may give a helpful indication of the molecular events underlying drug toxicity, while studies of the xenografts enables an understanding of the pharmacological events which will govern drug utility in the whole animal.

Although in 1985 the drug screening system of the NCI abandoned its *in vivo* tumour panel and adopted *in vitro* human cancer cell lines [Grever et al, 1992], the difficulty of converting *in vitro* data to parameters predictive of *in vivo* and clinical activity has not yet been resolved. The fact remains that many anticancer drugs - for example the platinum IV compound tetraplatin - were selected for further clinical development principally because of the activity that they demonstrated against murine tumours, and continued use of these non-human lines in screening systems is necessary to provide an essential historical reference in future drug development.

In addition to the L1210 murine leukaemia cell line and its resistant variants, other tumour models employed to screen the lead platinum compounds are the mouse plasmacytoma line ADJ/PC6 and human ovarian carcinoma cell lines. The importance of the ADJ/PC6 as a predictor of the clinical utility of platinum compounds was demonstrated by its use in the selection for clinical trials of carboplatin and iproplatin [Harrap, 1985]. An important feature of these murine cell lines is that they are freely interchangable from *in vitro* to *in vivo* and *vice versa*, allowing comparison of cytotoxic and pharmacologic mechanisms as outlined above.

At the Drug Development Section in the Institute of Cancer Research in Sutton (ICR), compounds which demonstrate activity against both resistant L1210 cell lines and the ADJ/PC6 *in vitro* may be tested in mice bearing

ADJ/PC6 xenografts. Thereafter, their cytotoxic activity against six human ovarian carcinoma cell lines is measured. These human ovarian cell lines are discussed in more detail in Chapter 5.4. They contain examples of tumours with both intrinsic and aquired resistance to platinum and have previously been ranked in order of their sensitivity to the four 'calibrating' platinum drugs cisplatin, tetraplatin, carboplatin and iproplatin (Hills et al,1989).

Using the cell lines as described above, several platinum compounds were screened for their activity as orally administerable drugs with antitumour activity and toxicity comparable to cisplatin and carboplatin. The advantages in having an orally delivered anticancer drug include more time at home for the patient, perhaps improving his quality of life, and less expensive in-patient treatment which is increasingly important in these cost-conscious times.

It has already been demonstrated that cisplatin, carboplatin and iproplatin have moderate antitumour activity after oral administration to mice carrying the PC6 tumour, but poor absorption necessitated delivery of much larger drug doses [Siddik et al, 1984; van Hennik et al,1989]. Significantly, oral administration ameliorated nephrotoxicity.

A compound which is to be absorbed following oral administration should be neutral and lipophilic since one of the most important pathways for

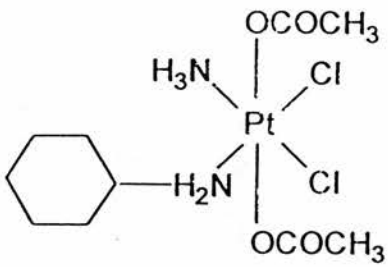
absorption is passive diffusion through the small intestine. The search for a new platinum drug must therefore be guided by a knowledge of the biochemical advantages conferred by particular structural groups. The interest aroused by the finding that the diaminocyclohexane (DACH) ligand conferred activity against L1210/cisplatin led to eventual clinical evaluation of compounds incorporating that structure, namely JM82, TNO6, oxaliplatin and tetraplatin, all of which were reviewed in Chapter 1.4.

The mixed amine compounds of general formula cis-Pt(II)$Cl_2(NH_2R)(NH_3)$ retain the principal structural features required for activity (cis arrangement of labile ligands, Pt in the +2 oxidation state) and the unsubstituted amine ligand serves to stabilise the DNA lesion through hydrogen bonding to the phosphate backbone of DNA [Sherman et al, 1985]. Many such compounds were developed and screened at the ICR for antitumour activity following oral delivery.

In this chapter, the results of activity and toxicity studies are presented for a series of six structurally related ammine/amine platinum IV dicarboxylates (Figure 5.1) which were tested against the L1210 sensitive and resistant lines *in vitro* and subsequently the ADJ/PC6 tumour *in vivo*. The six 'lead' compounds were then tested against the panel of human ovarian cancer cells *in vitro* in order that their activity in a human tumour might be assessed.

FIGURE 5.1

THE SIX 'LEAD' NOVEL PLATINUM COMPOUNDS



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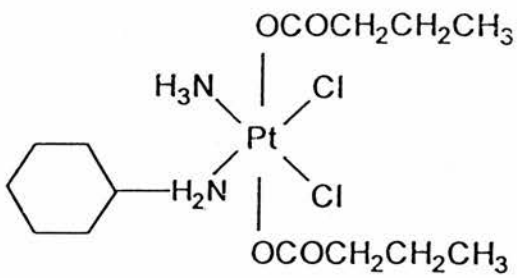

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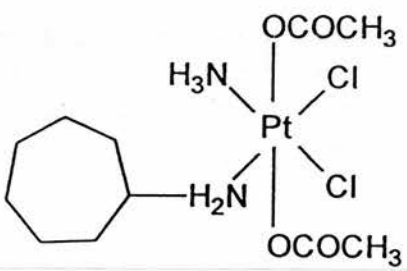

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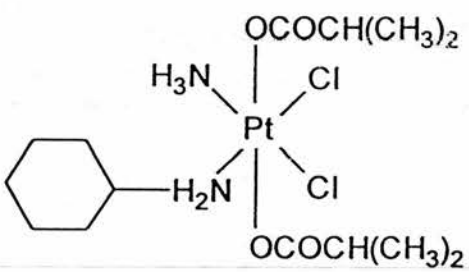

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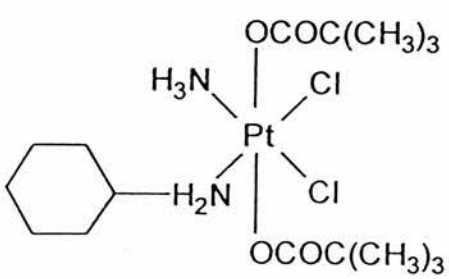

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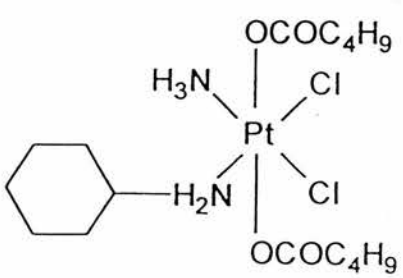

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5.2 Materials and methods.

5.2a Estimation of compound cytotoxicity in L1210 sensitive and resistant cell lines *in vitro*.

All cell lines were grown in supplemented RPMI 1640 medium as described previously. The starting concentration of cells was 5 x 10^4/ml, thus ensuring that cells were in the logarithmic growth phase. Test drug at five different concentrations was added to the cells and incubated at 37^{0}C for 48hours. The cells were then counted on a coulter counter and the IC50 was defined as that drug concentration which reduced the cell growth by 50%.

5.2b Estimation of compound potency against the ADJ/PC6 tumour *in vivo*.

Balb C mice of equivalent age had a 1mm^3 tumour implanted in the flank on day 1 of the experiment. On day 20, animals bearing tumours of comparable size were randomised to receive either drug (3 animals per dose level) or vehicle only (10 animals). Drug was administered after dissolution in arachis oil and sonication as a single dose by oral gavage directly into the stomach or by intraperitoneal (ip) injection. The animals were sacrificed on day 30 and tumours were dissected out from the treated and control groups and weighed. The relative effectiveness of each compound was then calculated through comparison of the therapeutic indices (see below).

5.2c Therapeutic index calculation.

The therapeutic index of a compound is calculated from a knowledge of its LD50 and ED90. The LD50 is that dose which proves lethal in 50% of the mice tested (Weil 1952). The ED90 represents the dose which is effective in reducing the tumour mass by 90%. Control mice are those which receive no drug. The tumour size in control mice is compared with the mean tumour weight for the three animals treated per dose of drug. The quotient of LD50/ED90 gives an indication of the relative selectivity of each compound tested. An increase in the toxicity (lower LD50) with no change in the drug's antitumour activity (ED90) would therefore result in a lower therapeutic index (TI).

5.2d Growth of the ovarian cell lines.

The six ovarian carcinoma cell lines were grown as monolayer cultures. All except 41M grew in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum, 50μg/ml gentamicin, 2.5μg/ml amphoteracin B, 2mM glutamine and 10μg/ml insulin with 0.5μg/ml hydrocortisone as growth factors in a 10% carbon dioxide, 90% air atmosphere. 41M cells were grown in a 1:1 mixture of DMEM and Hams F12 medium with the same additives. The lines established from xenografts (PXN94 and HX62) required control of stromal fibroblast overgrowth through selective detachment of fibroblasts using a 30 second incubation with 0.02% EDTA and the use of a feeder layer of lethally irradiated Swiss

mouse embryonic fibroblast 3T3 cells. Cellular contamination with mycoplasma was excluded by periodic checks using staining with Hoechst 33528 dye and examination under a fluorescent microscope.

5.e Preparation of cells for the Sulforhodamine Assay.

On day 0, each cell line was trypsinised, spun in a Centaur centrifuge at 1000g for 5 minutes and the pellet resuspended in 10ml supplemented DMEM. Cells were counted using a haemocytometer and resuspended at a concentration of 2.5×10^4/ml (excepting the 41M line which was set up at 5×10^4/ml). Using a Titertek Multidrop, 200μl cells were added to each well of a 96 well plate. Cells were then incubated in a CO_2 incubator at 37°C overnight to allow attachment.

The six lead compounds were prepared in solution on day 1. Drugs were dissolved in ethanol at 2mM (JM221, 272 and 273) or 1mM (JM216, 269 and 274) and serial dilutions were done in 2.5% ethanol, a concentration known to be non-toxic to the cells. The drug concentration range was 10μM - $2.5 \times 10^4\mu$M for the first three compounds and 5μM to $2.5 \times 10^4\mu$M for the latter three because of their lower solubility. Each drug was prepared at ten concentrations: 0.00025μM, 0.001μM, 0.0025μM, 0.01μM, 0.025μM, 0.1μM, 0.25μM, 1.0μM, 5μM and 10μM.

Drug was added to the wells in quadruplicate using a multipipetter. Control wells at either end of the plate were treated with 50μl of 2.5% ethanol/medium. The treated cells were then incubated for a further 96

hours.

5.2f The Sulforhodamine Assay.

The medium was decanted and the plates were gently immersed in ice-cold 10% trichloroacetic acid (TCA) and placed on ice for a minimum of 30 minutes. For cell lines prone to detachment (CH1 only), the TCA was poured off after 30 minutes and replaced with ice-cold methanol for 5 minutes. The fixative was then tipped off and the plates were washed gently with tap water five times. The plates were then placed inverted on tissue to dry.

Using the Titertek-Multidrop, 100μl of Sulforhodamine B (0.4% in 1% acetic acid) was added to 11 of the 12 rows. This was left for 15 minutes then tipped off and the plates washed 4 times with 1% Acetic acid. The plates were again inverted and left to air dry overnight.

The following day, 100μl 10mM Tris (unbuffered) was added to solubilise protein-bound SRB. The plates were set on a shaker for 5 minutes and their optical density read at 540nm.

The percentage inhibition of cell growth was then calculated, graphs drawn and the IC50's calculated.

Sulforhodamine detects the basic amino acids (mainly lysine) as detailed by Skehan and colleagues [Skehan et al, 1990].

5.3 Results.

Table 5.1 shows the in vitro cytotoxicity of the ammine/amine dicarboxylate dichlorides against the sensitive L1210, and L120/cisplatin, L1210/tetraplatin and L1210/carboplatin. Figures in parenthesis represent the -fold resistance compared with the sensitive line. All of the mixed amine complexes retain activity against the resistant L1210 cell lines with JM274 (R = cyclo-C6, $R_1 = C_4H_9$) the most potent - IC50's of 0.05 to 0.08uM.

Table 5.2 compares antitumour activity of oral and intraperitoneal platinum IV mixed amine complexes against ADJ/PC6 xenografts. Apart from JM221 (R = cyclohexyl, $R^1 = C_3H_7$) which was less potent when given orally (ED90 ip = 2.5mg/kg and po = 5.2mg/kg), the mixed amine compounds exhibit similar antitumour activity (ED90) when delivered by either route, but the LD50 is substantially increased following oral administration consistent with reduced toxicity (eg.JM274 where R = cyclohexyl, $R_1 = C_4H_9$ has LD50 ip = 14 and po = 1120). The TI is therefore increased with oral administration of all six compounds (eg. JM269 where R = cycloheptyl, $R_1 = CH_3$ has TI ip = 6.1 and po = 269). The data for cisplatin are added for comparison at the end of the Table 5.2. It can readily be seen that although there is more potency (low ED90 of 0.6mg/kg) following ip delivery of cisplatin, oral administration is significantly more toxic (LD50 = 14mg/kg versus the lowest of 170mg/kg for JM272 where R = cyclohexyl, $R_1 = (CH_3)_2$) and there is a large difference in the TI (6 for cisplatin compared with 59 -

TABLE 5.1

In vitro **cytotoxicity of lead ammine/amine dicarboxylate dichlorides against sensitive and resistant L1210 cell lines.**

Compound JM No.

IC50 (μM)

Cell Line	216	221	272	273	274
L1210/s	1.2	0.06	0.14	0.08	0.05
L1210/cis	1.5	0.11	0.1	0.07	0.08
(10)	(1.3)	(1.8)	(0.7)	(0.9)	(1.6)
L1210/tetra	1.4	0.06	0.07	0.04	0.05
(34)	(1.2)	(1.0)	(0.5)	(0.5)	(1.0)
L1210/carbo	1.6	0.12	0.21	0.11	0.08
(13.7)	(1.3)	(1.5)	(1.5)	(1.4)	(1.6)

Figures in parenthesis represent - fold resistance compared with the parent sensitive cell line.

TABLE 5.2

Antitumour activity of the dicarboxylate dichloride Pt IV ammine/amines against ADJ/PC6 tumour xenografts.

JM' No	Intraperitoneal (ip) mg/kg			Oral (po) mg/kg		
	LD50	ED90	TI	LD50	ED90	TI
216	30	6	5	330	5.8	57
221	15.5	2.5	6	280	5.2	54
269	71	11.7	6	2690	10.4	269
272	21	2.9	7	170	2.9	59
273	30	5.2	6	670	3.7	181
274	14	5	3	1120	3.6	311
Cisplatin	11.3	0.6	19	14	2.4	6

3 animals treated per dose level

10 animals were controls - received vehicle only.

lowest value of all compounds - for JM272).

Activity of JM216, 221, 269, 272, 273 and 274 against six human ovarian lines *in vitro* (as measured by the Sulforhodamine assay) is shown in Figures 5.2 to 5.7 with a summary in Table 5.3. The most resistant ovarian carcinoma cell line in early experiments with 'calibrating' platinum compounds was SKOV3. In every case the most potent compound was JM274 (R = cyclohexyl, $R_1 = C_4H_9$) which had the lowest IC50 concentration in all six cell lines. JM273 (R = cyclohexyl, $R_1 = (CH_3)_3$) also demonstrated excellent antitumour activity. The least effective compounds were JM216 (R = cyclohexyl, $R_1 = CH_3$) and JM269 (R = cycloheptyl, $R_1 = CH_3$).

Figure 5.8 demonstrates the relative sensitivity of the six ovarian cell lines to the six lead compounds with the most resistant line HX62 taken as standard (RS1). In these experiments, CH1 was the most sensitive cell line. Comparison of this graph with that delineating relative sensitivities of the human ovarian cell lines to the four 'calibrating' platinum drugs (Figure 5.9) belies the much greater potency of the mixed amine dicarboxylate compounds. Figure 5.10 compares the IC50's of the six lead compounds and cisplatin, carboplatin and tetraplatin against the most sensitive ovarian cell line CH1 and the most resistant line HX62. It is quite clear that the new compounds are generally much more cytotoxic against the human ovarian cancer cells *in vitro*.

Figure 5.2 IC50 of the six JM lead compounds versus CH1.

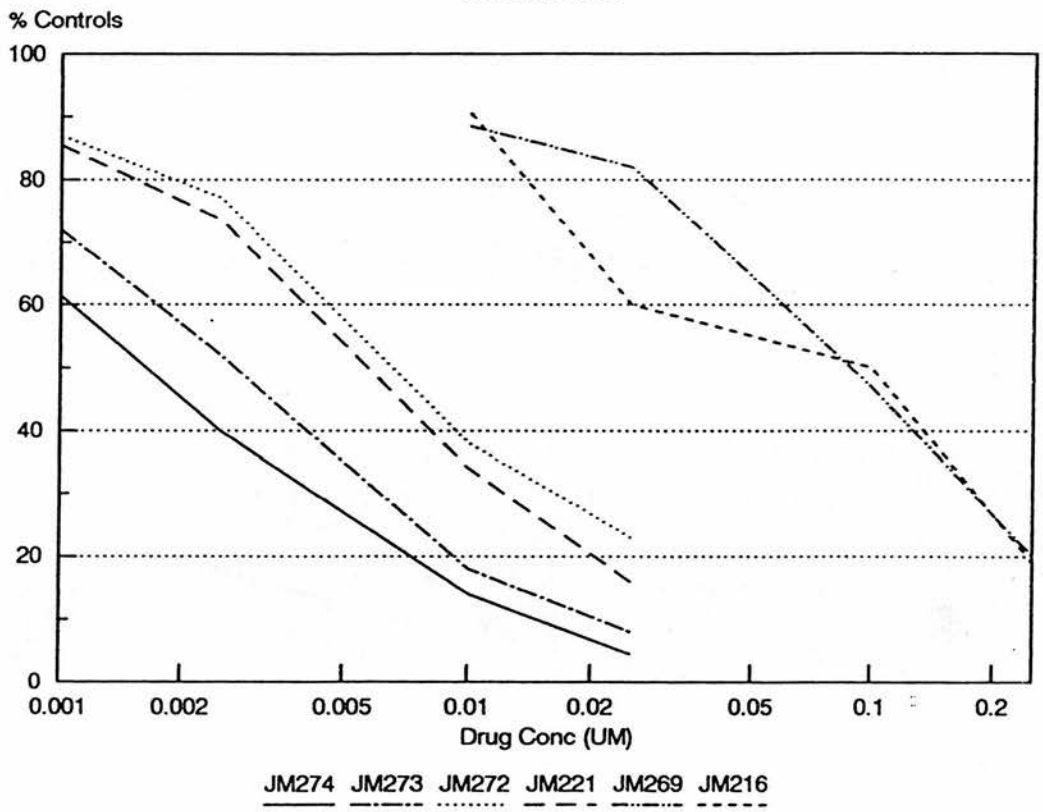


Figure 5.3 IC50 of the six JM compounds versus 41M

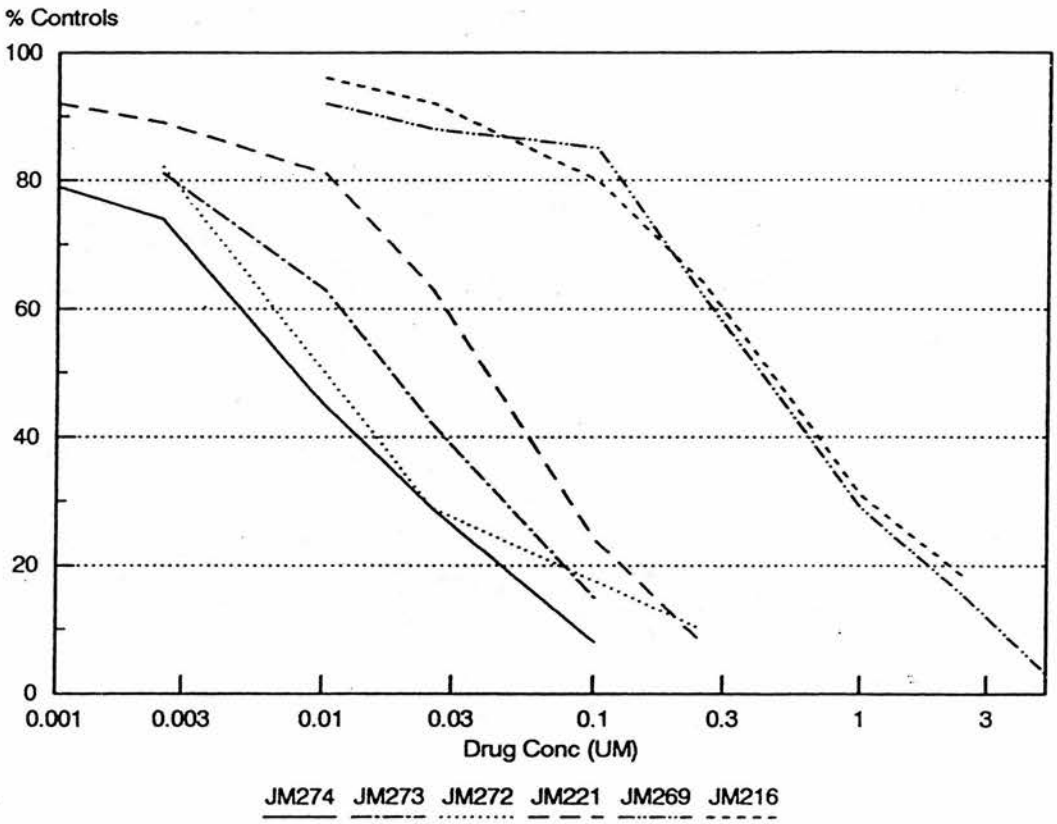


Figure 5.4 IC50 of the six JM lead compounds versus PXN94.

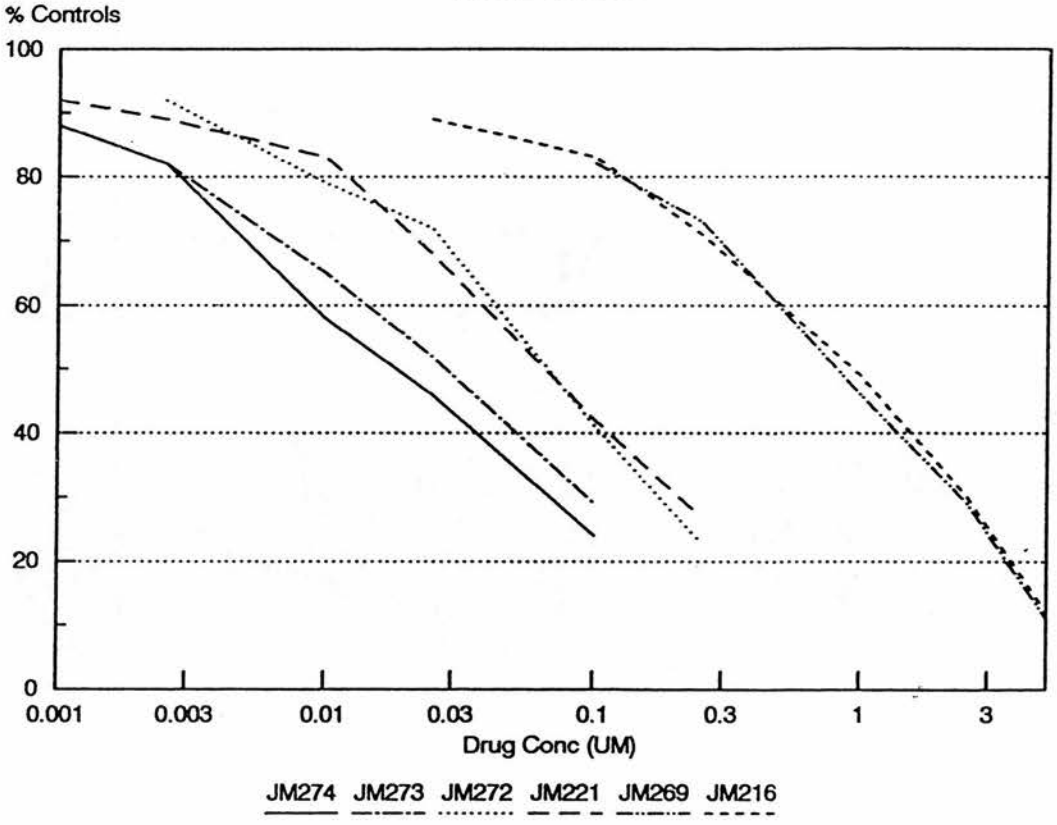


Fig 5.5 IC50 of the six JM lead compounds versus OVCAR3.

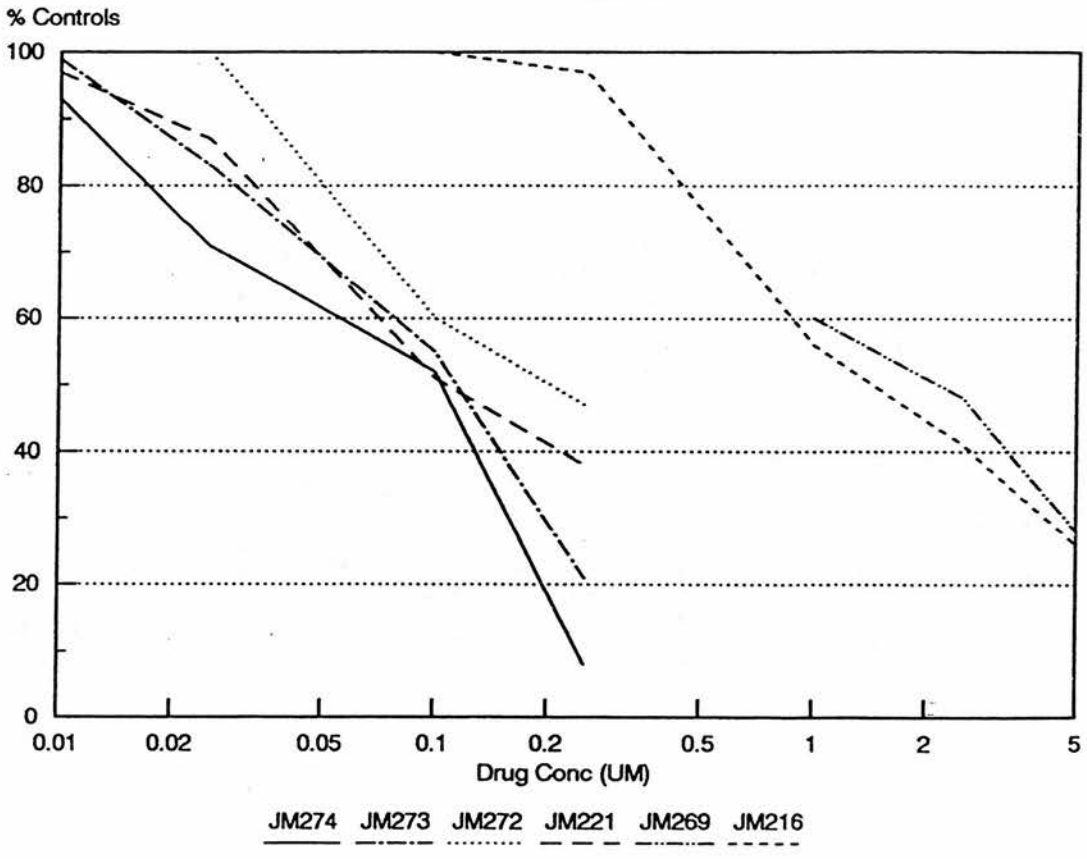


Fig 5.6 IC50 of the six JM lead compounds versus HX 62.

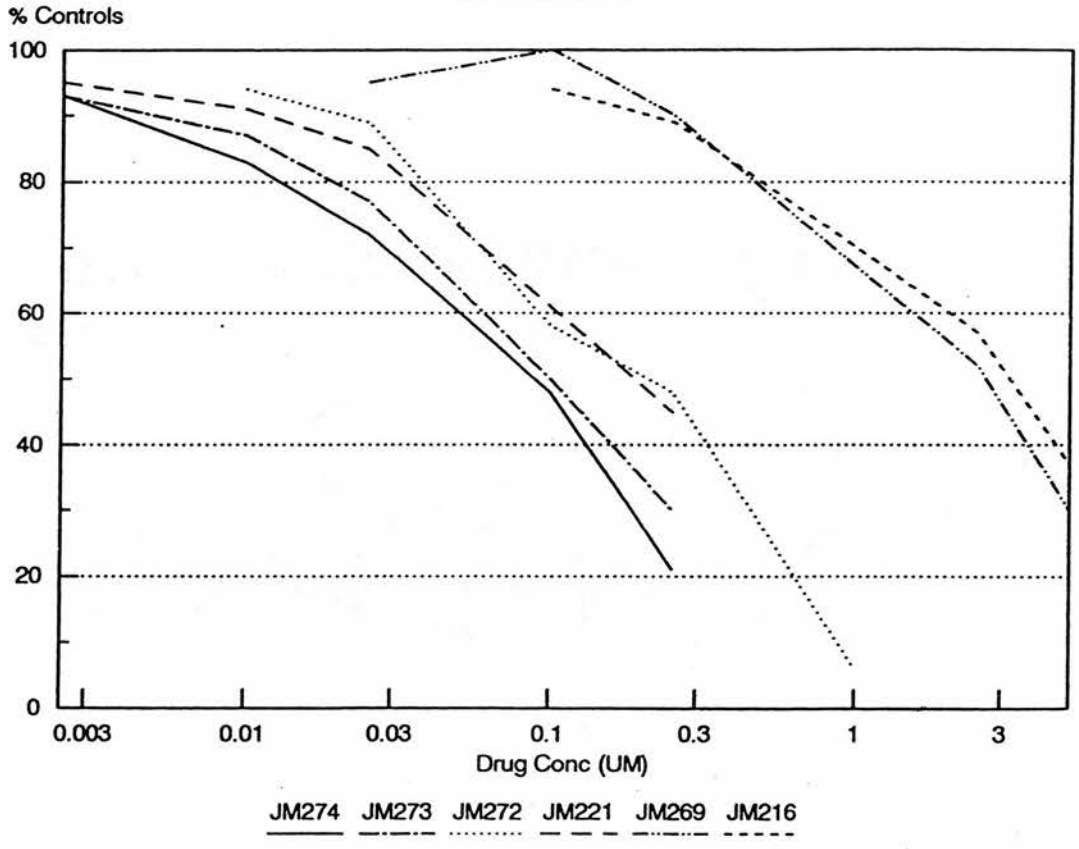


Fig 5.7 IC50 of the six JM lead compounds versus SKOV 3.

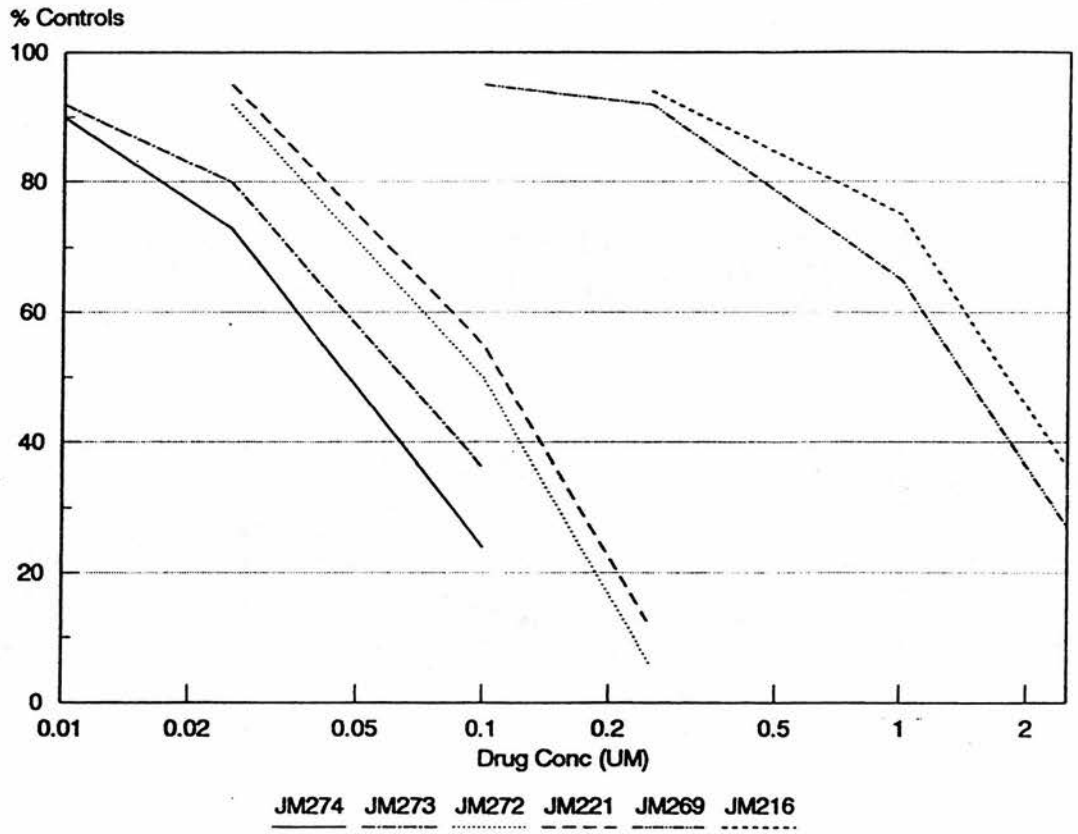


TABLE 5.3

IC50 of the six lead compounds and three 'conventional' platinum drugs against the six human ovarian cell lines *in vitro*

	Compound JM No.								
	216	221	269	272	273	274	Cisplatin*	Carboplatin*	Tetraplatin*
Cell line	IC50μM								
CH1	0.1	0.056	0.088	0.0065	0.0026	0.0016	0.13	0.59	0.28
4IM	0.45	0.04	0.41	0.056	0.017	0.0078	0.04	0.23	0.26
PX94	0.94	0.064	0.08	0.064	0.027	0.019	0.89	3.2	0.13
HX62	3.2	0.2	2.7	0.19	0.1	0.086	2.4	10.3	2.0
OVCAR3	1.4	0.1	2.0	0.2	0.115	0.1	0.2	0.75	1.0
SKOV3	1.8	0.11	1.4	0.1	0.064	0.048	4.1	15.6	14.4

* Continuous exposure for 96 hours

Figure 5.8 Relative sensitivity of ovarian cell lines
(most resistant line HX62 = standard 1)

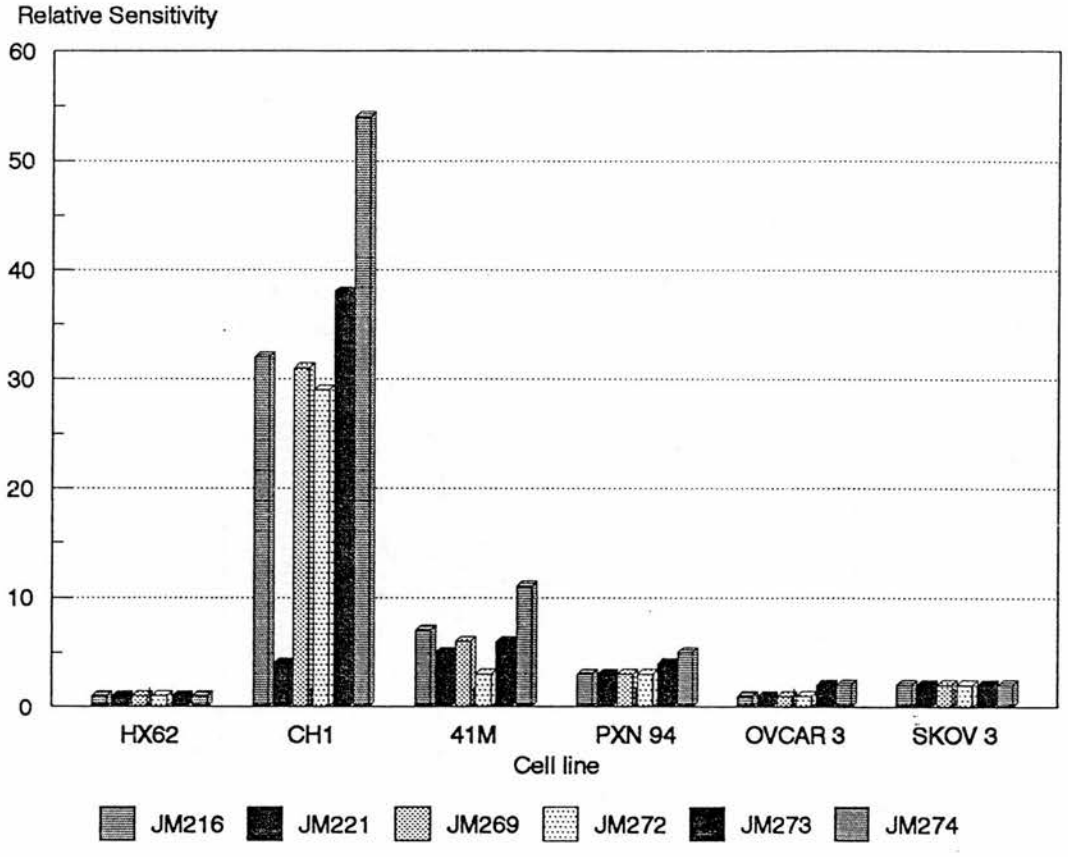


Figure 5.9 Relative sensitivity of ovarian carcinoma cell lines (with SKOV-3 line = standard 1)

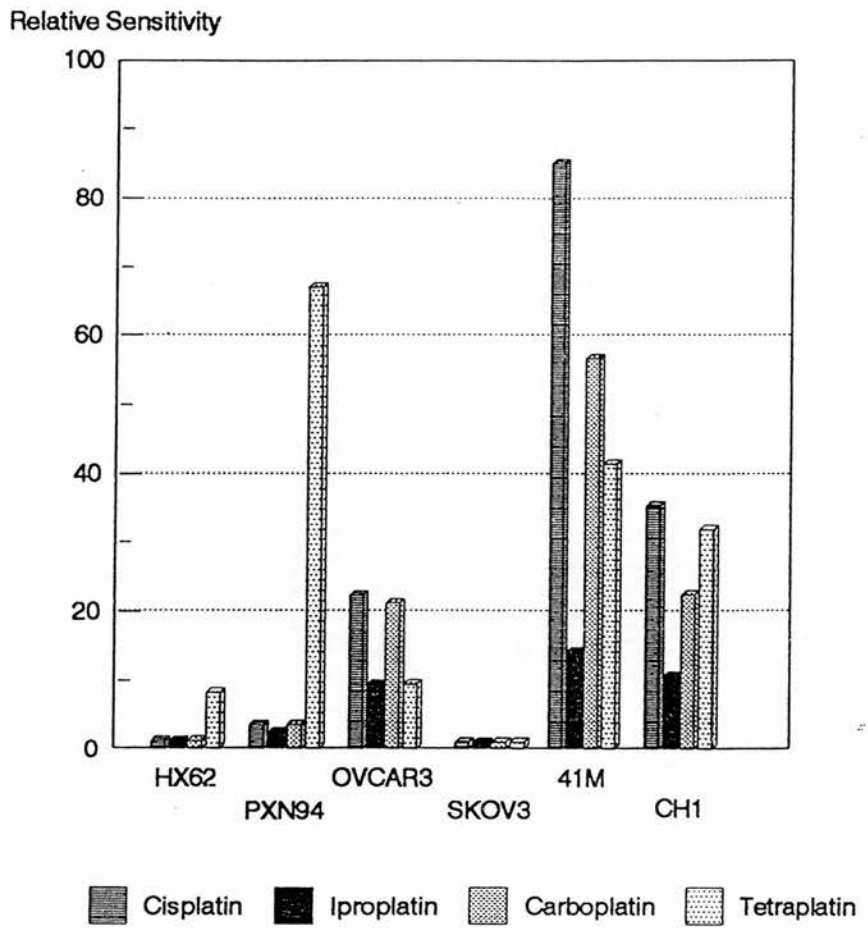
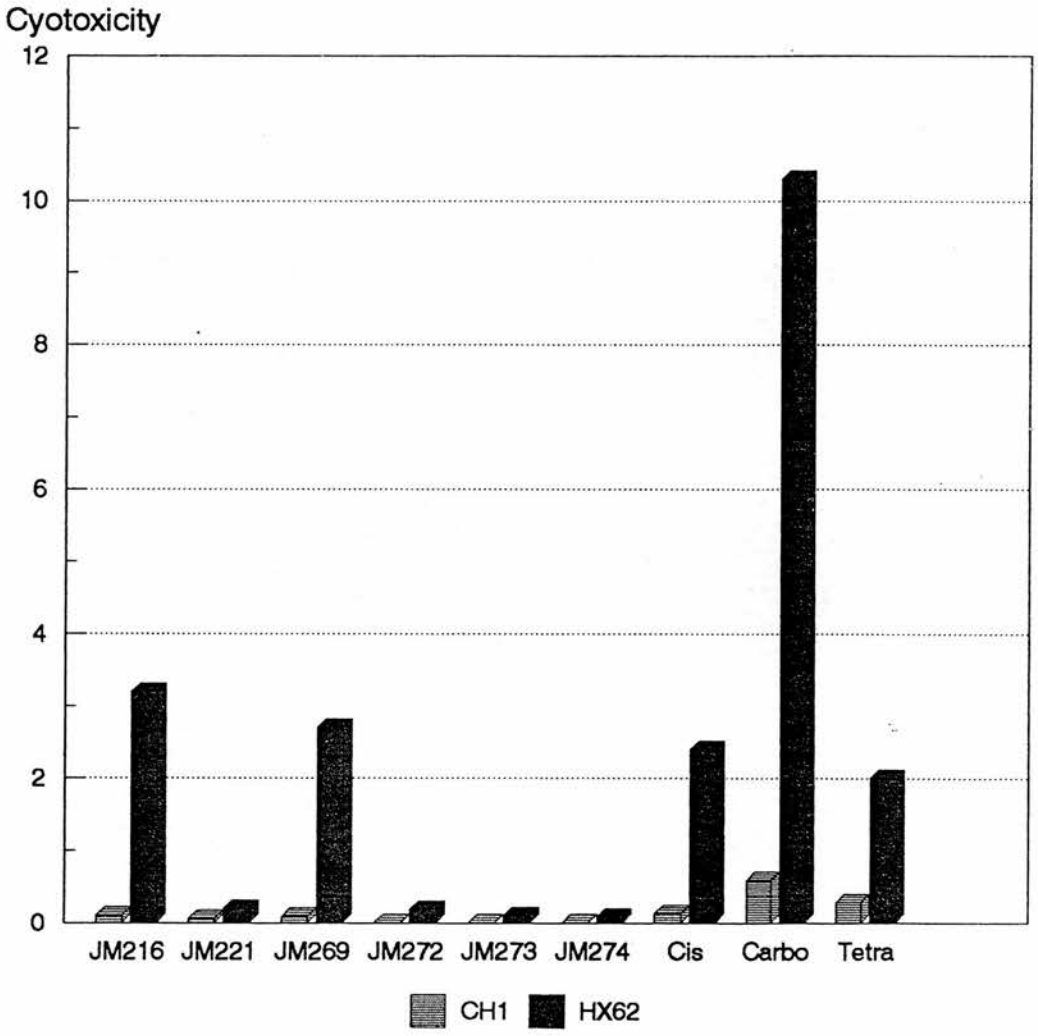


Figure 5.10 Comparison of cytotoxicity of the six 'lead' compounds and the 'calibrating' platinum drugs against the most sensitive (CH1) and resistant (HX62) human ovarian cancer cell lines.



5.4 Discussion.

All of the six platinum IV transdihydroxydichloro-ammine/amine compounds tested retain activity against all of the L1210 platinum resistant lines (Table 5.1). This finding was believed to be relevant in the search for a new and more active platinum based anticancer drug and warranted further investigation of the compounds *in vivo*.

In the ADJ/PC6 tests *in vivo*, the mixed amine dicarboxylates exhibited comparable antitumour potency when administered either orally or by the intraperitoneal route (ip), but acute toxicity (LD50) was substantially reduced by oral delivery (Table 5.2). In addition, the therapeutic index (LD50/ED90) was greater when the compounds were given orally because of more selective antitumour activity shown by a fall in the ED90 and an increase in LD50.

The six human ovarian cancer cell lines used in experiments detailed in this thesis are well established in culture systems. OVCAR3 [Hamilton et al, 1983]) and SKOV3 [Fogh et al, 1977] were obtained from the American Type Culture Collection. 41M was established by AP Wilson at the Oncology Research Laboratory in Derby City Hospital, UK [Hills et al, 1989]. CH1, PXN94 and HX62 were established in the tissue culture laboratories at the ICR in Sutton; CH1 was developed from the ascites of a woman with ovarian carcinoma and the other two were from human ovarian xenograft

lines grown in female nude mice. Calibration of all six lines against cisplatin, tetraplatin, carboplatin and iproplatin has been performed and the cell lines provide a useful ranking system because of their different sensitivities to these four drugs [Hills et al, 1989] (Figure 5.9). The resistant patterns of the cell lines represent both innate and aquired resistance to platinum chemotherapy.

When the relative sensitivities of the ovarian cell lines to the six lead JM compounds are plotted (Figure 5.8), in contrast to the plot shown in Figure 5.9 there is very little differential sensitivity. CH1 was again the most sensitive cell line. The most active compound was JM274 (R = cyclohexyl, $R_1 = (C_4H_9)$ which was 16 times more potent against the CH1 - IC50 = 0.0016uM - than was JM221 (R = cyclohexyl, $R_1 = C_3H_7$) - IC50 = 0.056uM (Table 5.3). This result suggests that potency is increased by lengthening the axial ligand (R_1).

The compounds JM274 and JM273 vary only in the axial group which is straight (C_4H_9) for JM274 and branched ($(CH_3)_3$) for JM273. In the experiments on tumour-bearing mice (Table 5.2), the TI was higher for JM274 at 311 compared with 181 for JM273. The result was effected mainly by the reduced toxicity of JM274 (LD50 = 1120mg/kg versus 670mg/kg). The IC50's of these compounds against the human ovarian cell lines were similar except against 41M where the results were 0.0078uM

(JM274) and 0.017uM (JM273) (Table 5.3).

The number of carbon atoms in the ring on the R group appeared to be immaterial since on comparison of the cytotoxicity of JM216 (R = cyclohexyl) with JM269 (R = cycloheptyl) where in both cases the R_1 group is a simple methyl (CH_3) the IC50's against the ovarian cell lines were eqivalent (Table 5.3). However in the *in vivo* experiments the antitumour activity in ADJ/PC6 tumour-bearing mice demonstrated a clear advantage for the cyclocycloheptyl compound JM269: The low toxicity following oral administration (LD50 = 2690mg/kg) contrasted with the result for JM216 (LD50 = 330mg/kg) with the respective TI's 269 and 57 (Table 5.2). Note from the data detailed above that the TI of the cyclohexyl longer axial chain compound JM274 was comparable to that of JM269 (311).

In summary, these experiments demonstrated optimum cytotoxic activity with longer chain axial ligands. Equivalent activity was not obtained through shortening the axial chain and substituting the cyclohexyl group at R for a cycloheptyl moiety.

In comparing the cytotoxic activity of the six PtIV dicarboxylatedichloro mixed amine compounds with the established drugs cisplatin, carboplatin and tetraplatin, very little cross-resistance is seen (Figure 5.10), and these data along with the potency data from the ADJ/PC6 tumour-bearing mice

emphasise the need to explore further the novel compounds as orally delivered anticancer drugs.

In the following chapter, pharmacological testing allows further analysis of the six "lead" compounds.

CHAPTER 6

PHARMACOKINETICS AND HAEMATOLOGICAL TOXICITY STUDIES ON LEAD COMPOUNDS

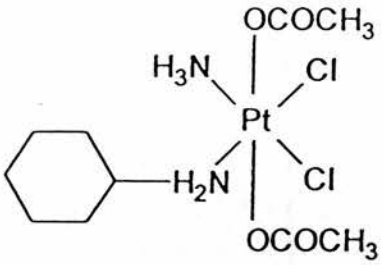
6.1 Introduction.

The advantages in having a platinum based drug which could be delivered orally have already been stated. In addition to the practical considerations, there may be an improvement in the spectrum of activity and in the toxicity profile of a platinum drug which could be given at low dose for longer periods of time. Treatment with a 5-day infusion of cisplatin has been shown to reduce nephrotoxicity without associated diminution of antitumour activity [Salem et al, 1984]. It has previously been shown that cisplatin and carboplatin possess moderate antitumour activity against the ADJ/PC6 plasmacytoma when given orally [Siddik et al, 1984]. Nephrotoxicity was 20% with oral administration of cisplatin compared with a 6-fold increase in blood urea in all animals treated by the intravenous (IV) route. The reduced nephrotoxicity may have been related to the fact that these drugs are poorly absorbed when they are delivered by the oral route.

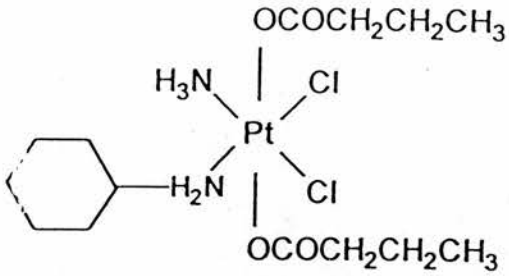
In the previous chapter, the screening panels were seen to be effective in selecting several compounds which showed good antitumour activity against the sensitive and resistant tumours in vitro and in vivo. The TI's of the dichlorodicarboxylate ammine/amine platinum IV complexes (structures Figure 6.1) measured in the ADJ/PC6 after an oral dose of the

FIGURE 6.1

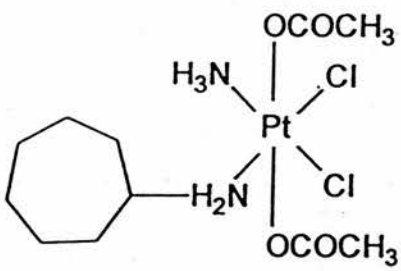
The six 'lead' novel platinum compounds



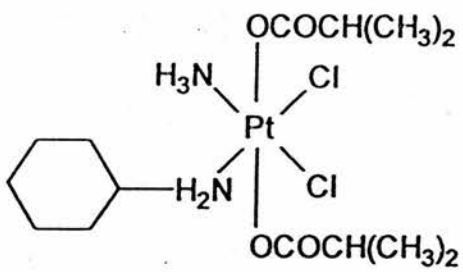
JM216



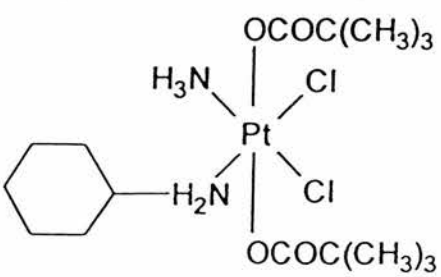
JM221



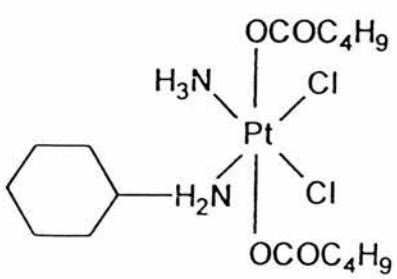
JM 269



JM272



JM273



JM274

"dicarboxylates" ranged from 50 to over 300, compared with 6 for cisplatin, as shown in Chapter 5. The six lead compounds selected from previous experiments demonstrated good antitumour activity with relatively low toxicity, therefore it was important to define their pharmacological profiles and to ascertain their absorption characteristics.

Oral bioavailability is defined as the rate and extent to which an active drug substance is absorbed and becomes available to the general circulation. Bioavailability is not, therefore, simply a property of the drug itself but also depends on the formulation in which the drug is delivered. For a drug to be bioavailable, it must reach the general circulation intact. The important difference between absorption and bioavailability becomes apparent when one considers that a drug, once absorbed, may undergo metabolism or elimination during its passage through the gastrointestinal (GI) tract, liver and lungs. If the drug is subjected to first pass metabolism in any of these tissues, a portion of the absorbed drug will be lost and consequently less of the absorbed dose will be "bioavailable". A drug with low bioavailability which also has a narrow therapeutic range may result in no therapeutic benefit if there is variability in the oral absorption. Conversely, toxicity may result should a large proportion of the drug enter the circulation in its active form [Sietsema, 1989].

GI absorption is assessed by comparing iv with oral delivery and also by measuring blood and urine levels after drug administration. The faecal excretion should also be known since drug recovered in the faeces

represents the unabsorbed fraction. The enterohepatic circulation is the system by which the drug is absorbed from the intestine into the liver, excreted into the bile, and re-excreted into the intestine prior to clearance either in the faeces or the urine. Delay in faecal excretion beyond 96 hours may signify extensive enterohepatic circulation of a drug.

In the study of platinum complex absorption, total and free platinum blood levels are measured. The area under the plasma concentration v. time curve (AUC) is a measure of plasma platinum concentration variation with time and enables calculation of the elimination half lives of both total and free platinum. The total platinum concentration represents the sum of the protein bound and free platinum whereas the free fraction is the active platinum.

The pharmacokinetic profile (ie: absorption, distribution, metabolism and excretion) of a drug characterises and identifies a safe dose for administration and also defines doses at which toxicities occur. In this chapter, the mouse model is used to establish the pharmacokinetic profiles and the haematological toxicity of the six lead dicarboxylate complexes.

Establishment of the murine LD10 dose allows determination of the starting dose level for human Phase I studies, normally 10% of the mouse LD10.

6.2 Materials and methods.

6.2a Mice:

Non-tumour bearing female Balb-C mice were used for all experiments. Animals were housed in metabolism cages (Jenson Scientific, UK) for 96 hours with free access to food and water. Mice were weighed daily and the percentage weight loss tabulated. Blood sampling was by the axillary vessels and was performed under halothane anaesthesia prior to sacrifice by cervical dislocation.

6.2b Compounds and doses.

Structures of the six test compounds are shown in Figure 6.1. All platinum compounds were synthesised and donated by the Johnson Matthey Technology Centre (Sonning Common, UK) and Johnson Matthey International (USA) as part of a collaborative project. Drugs were suspended in arachis oil (Sigma,UK) and sonicated briefly (<10 sec at 16 Hz) to produce a fine suspension for immediate oral administration. Drug suspensions were delivered to mice by oral gavage using an animal feeding needle (Poffer and Sons, New York, USA) at a dose of 100umoles/kg (19.5mgPt/kg) in an injection volume of 10ml/kg.

6.2c Identification of LD10.

The oral LD10 (lethal dose in 10% of mice) or maximum tolerated dose (MTD) of each drug was established using as a guide the initial PC6 data in tumour-bearing mice (courtesy of P. Goddard). Five female non-tumour

bearing Balb C mice were treated with one of five doses of each drug as detailed below:

Compound	Doses administered (mg/kg)
JM216	200, 250, 300, 350, 400
JM221	200, 250, 300, 350, 400
JM269	1000, 2000, 3000
JM272	100, 140, 180, 220, 260
JM273	100, 200, 400, 600, 800, 1000, 1200
JM274	400, 600, 800, 1200, 1600

The weight of each mouse was recorded daily over a period of 28 days and the percentage weight loss recorded. Sick mice were sacrificed and any particular features thought to contribute to their illness were noted.
The LD10 was estimated as the dose incurring approximately 20% body weight loss and/or 10% lethality in the mice.

6.2d Pharmacokinetics of orally administered Pt IV dicarboxylates.

The absorption of the lead ammine/amine platinum IV dicarboxylates was established in mice through measurement of the plasma levels of total, free and reversibly bound platinum following equimolar oral doses (100μmoles/kg) of each of the six Pt IV complexes. Three mice per time point were treated with each drug. Blood and plasma collection methods are described below.

The terminal half lives of the platinum were compared for the compounds

to enable evaluation of an optimal dosing frequency, with the aim of maximising activity without incurring unacceptable toxicity.

AUC values (0-24 hours for total platinum and 0-4 hours for "free" platinum) were determined by the trapezoidal rule. A two compartment model ($C = A \exp(-at) + B \exp(-bt)$) was used to determine the terminal half life of total platinum and a single compartment model ($C = A \exp -(at)$) for ultrafilterable platinum. The weighting factor for the curves (estimated by least squares linear regression) was $1/(Y + Yhat)^2$.

6.2e Pharmacokinetics: Blood and plasma collection.

Mice were fasted 18 hours prior to drug administration to optimise the absorption kinetics and overcome interactions with stomach contents. Blood sampling was performed at 15', 30', 1, 2, 4, 7, 24, 48, 72 and 96 hours following drug administration.

1.0 ml blood samples were collected in 1.5ml microfuge tubes and spun for 3 minutes at 1000rpm in a Beckman Microfuge (model B) to obtain the plasma. Plasma was then divided into

a) 50μl + 100μl water - for analysis of total platinum

b) 25μl + 50μl ice cold methanol; the combined free and reversibly bound platinum was detected in the soluble fraction following addition of methanol at 4^{0}C, standing the sample on ice for 10 minutes then spinning it in the microfuge for 1 minute. The precipitate was discarded.

c) free platinum in the plasma was determined following ultrafiltration in a Europa centrifuge at 3000rpm, 4^{0}C for 30 minutes using Amicon CF50A centrifree membrane cones as described by Litterst (Litterst 1976(b)).

Samples were stored at -20^{0}C until analysis when they were diluted (as detailed above) and analysed for platinum by flameless atomic absorption spectrophotometry using a Perkin-Elmer HGA 700 atomic absorption spectrophotometer.

Results are expressed as μgPt/ml plasma.

6.2f Excretion of platinum.

The excretion of each compound was measured as recovery of platinum in the faeces and the urine of Balb C female mice. Three mice were treated with each drug at 100μmoles/kg (19.5mgPt/kg). Faecal pellets were collected and weighed at 24 hour intervals. Urine was collected daily in labelled, pre-weighed microfuge tubes. The metabolism cage was rinsed daily with 10ml water and the resultant wash was collected in a stoppered Nunc tube.

6.2g Solubilisation and analysis of faecal samples.

Samples were weighed (each approximately 300mg), placed in 20ml pyrex pots and 2ml concentrated fuming nitric acid were added to each pot. The samples were left to digest in a fume cupboard overnight. The nitric acid was boiled off by placing the pots on a hot metal block and heating at 120^{0}C for 1 hour. When 100-200μl remained, 2ml 1.0N Hydrochloric acid were added and the boiling repeated, after which 2ml 0.1N Hydrochloric acid were added and boiled off. A further 2ml 0.1N Hydrochloric acid were then added and the mixture warmed gently. The contents were pipetted into 10ml stoppered Nunc tubes. The original pot was washed with 2ml

0.1N HCl which was decanted into the Nunc tube and made up to a final volume of 5ml.

The samples were diluted as tabulated below prior to being analysed by flameless atomic absorption spectrophotometry (FAAS), model IL655:

Sample time (hours)	Fold dilution (in 2.5ml 0.1N HCl)	Deposition time (seconds)
24	251	5
48	101	5
72	51	25
96	51	25

Standards were prepared at 196ng Pt/ml (50μl of 10μg/ml Pt standard to 2.5ml 0.1N HCl) for the 5 second deposition time and 49ng Pt/ml (50μl of 2.5μg/ml Pt standard to 2.5ml 0.1 HCl) for a deposition time of 25 seconds. The % dose excreted in faeces was assessed for individual mice.

6.2h Analysis of urinary excretion.

On completion of the experiment the microfuge tubes were reweighed and the volume of urine was calculated. The volume of the washes was made up to 10ml with water. Urine samples were diluted as tabulated below prior to being analysed by FAAS:

Sample time (hours)	Fold dilution (in 2.5ml 0.1N HCl)	Deposition time (seconds)
24	251	5
48	251	25
72	101	25
96	101	25

The 24 hour washes were diluted 1:3 and later washes were measured neat. Deposition time was 3 seconds in all cases.

Standards were prepared as for the faeces samples (outlined above).

The % dose excreted in urine was assessed for individual animals at each time point.

6.2i Assessment of haematological toxicity.

The haematological toxicity of each compound was measured in Balb C female mice following an oral dose of the LD10, 0.5 LD10 and 0.25 LD10. Three mice were dosed at each level for each of the six drugs, and controls received oil only or no treatment. All drugs were made up in arachis oil and administered by oral gavage. Sampling time points were days 1, 2, 4, 7, 10, 14, 21 and 28. Blood samples (0.5ml) were placed in heparinised tubes and delivered to the Haematology Laboratory, Royal Marsden Hospital, Sutton for analysis of the full and differential blood count. Results are expressed as a mean percentage of control cell counts +/- standard error. The nadir and temporal profile of white cell, haemoglobin and platelet counts were assessed for all compounds.

6.3 Results.

Table 6.2 shows the LD10 calculated from the nearest test dose causing approximately 20% weight loss in 5 mice. For compounds JM216, 221, 272 and 274 the lowest doses tested were closest to the LD10 and final dose selected for the experiments. The selected dose for JM269 was taken as the point between 1g/kg and 2g/kg because of the weight losses of 23% and 27% (lowest levels) for these two wide-ranging test doses. The LD10 for JM273 in PC6 tumour-bearing mice was around 600mg/kg and initial experiments in the non-tumour-bearing Balb-C mice initially used this as the mid dose. However, there was unacceptable toxicity and the experiment was repeated using 100, 200 and 400mg/kg. The final dose selected as the LD10 was 150mg/kg.

Figures 6.2 to 6.7 represent plasma concentrations of total and free platinum in mice following dosing with 100μmoles/kg of each test compound. For each Pt IV dicarboxylate, plasma concentrations are expressed as μgPt/ml and plotted against time. The peak plasma levels occurred very rapidly - between 15 and 60 minutes - with a small second peak at 4 or 7 hours. These findings are consistent with very rapid absorption following oral administration. The peak plasma concentrations were between 1.94μgPt/ml (JM274 R=cyclo-C6, $R_1 = OCOC_4H_9$) and 15.1μgPt/ml (JM273 R=cyclo-C6, $R_1 = OCOC(CH_3)_3$).

TABLE 6.2

Selection of the murine LD10* for lead compounds.

JM no.	Body weight** (% original)	Nearest dose***	LD10
216	75+/-4	250mg/kg	200
221	75+/-7	200mg/kg	150
269	74+/-9	2g/kg	}1.5
	85+/-8	1g/kg	
272	87+/-10	100mg/kg	100
273	82+/-13	200mg/kg	150
274	91+/-4	400mg/kg	400

* LD10 dose = dose incurring 20% weight loss+/-10% lethality
** 5 mice treated at each dose level
*** 5 doses per drug

Figure 6.2 JM216 Total and Free Pt concentration (μg/ml) following 100 μm/Kg Po

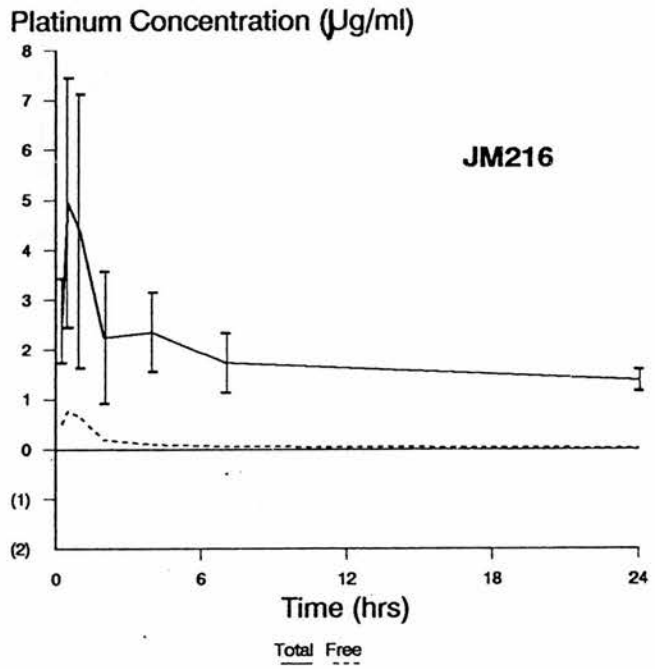


Figure 6.3 JM221 Total and Free Pt concentration (μg/ml) following 100 μm/Kg Po

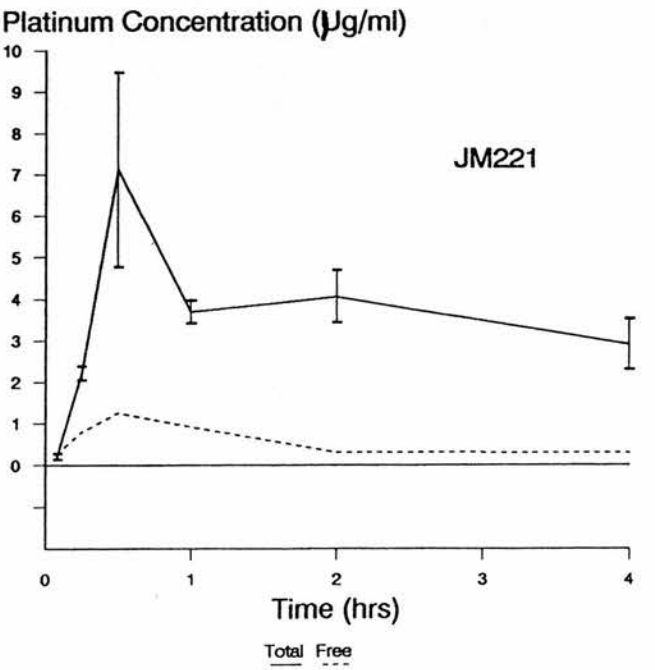


Figure 6.4 JM269 Total and Free Pt concentration (μg/ml) following 100 μm/Kg Po

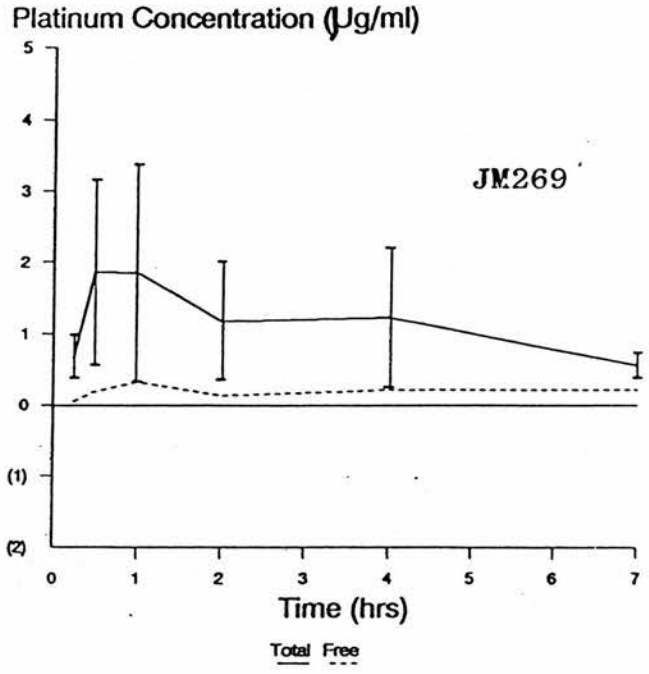


Figure 6.5 JM272 Total and Free Pt concentration (μg/ml) following 100 μm/Kg Po

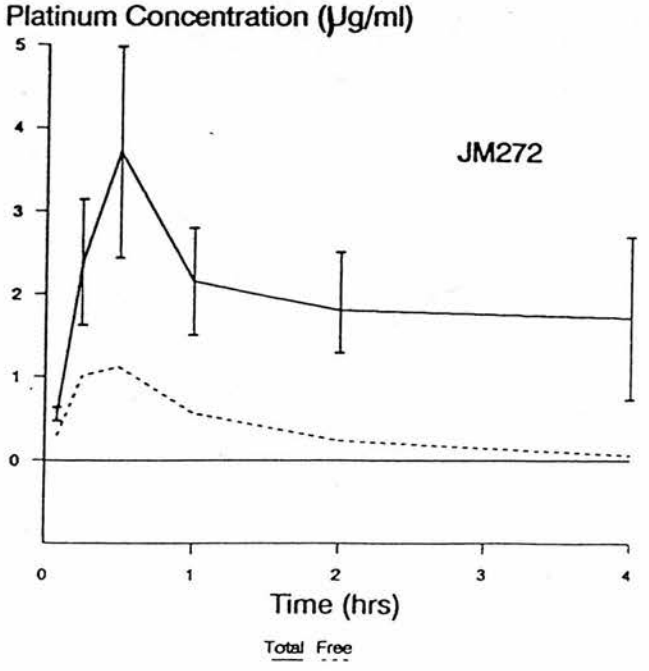


Figure 6.6 JM273 Total and Free Pt concentration (μg/ml) following 100 μm/Kg Po

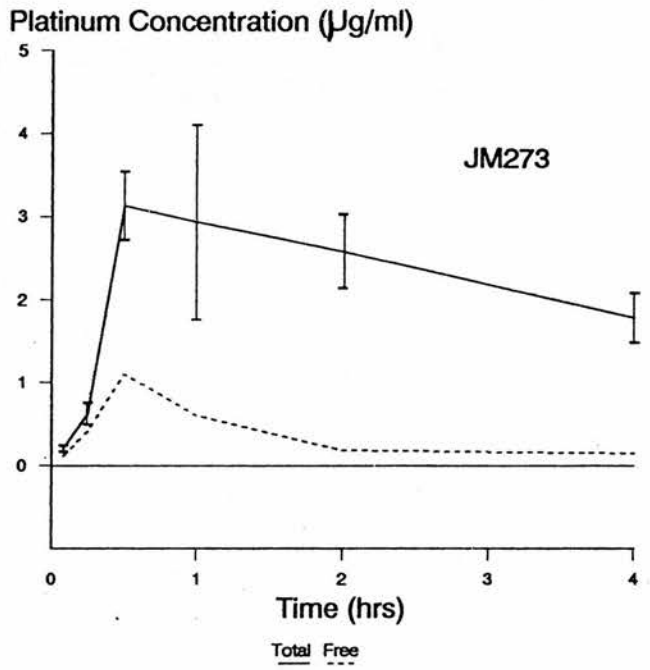


Figure 6.7 JM274 Total and Free Pt concentration (μg/ml) following 100 μm/Kg Po

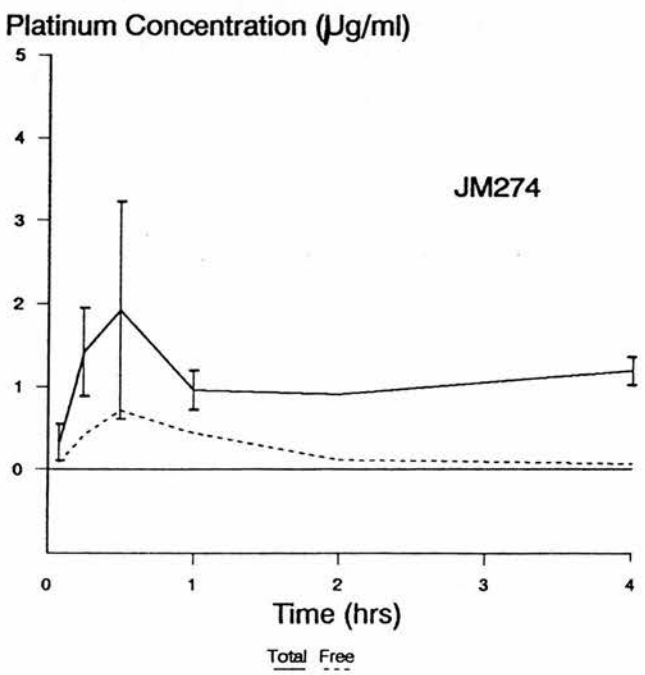


Table 6.3 summarises the plasma peak platinum concentrations, total and free platinum versus time concentrations (AUC data) and details the plasma half life for each of the compounds. JM269 (R = cyclo-C7, $R_1 = CH_3$) showed low absorption at the 96 hour time point (1.9μgPt/ml), as did JM274 with its long R_1 straight chain (C_4H_9) (1.94μgPt/ml). The highest plasma concentration was achieved with oral JM221 (R = cyclo-C6, $R_1 = CH_3$) at 120μgPt/ml/hr and there was also a high free platinum concentration (3.2μgPt/ml/hr) at 7 hours after dosing. Interestingly, the plasma half life of JM221 was second shortest at 18.8 hours for the total yet the free fraction demonstrated a t1/2 close to that of the other compounds (3.14 minutes). JM273 was absorbed quite well and the relatively high concentration of free platinum (1.43μgPt/ml/hr) had a remarkably long half life (35.5 minutes) even although the total platinum was rapidly cleared, demonstrating the shortest half life at 17.3 hours. JM216 was well absorbed (AUC for total platinum 108μgPt/ml/hr) but there was a disproportionately low free platinum concentration (0.7μgPt/ml/hr).

The faecal excretion data are shown in Table 6.4. At the 24 hour time point, the faecal excretion of the six compounds ranged from 14-41%. At 96 hours JM216 and JM269 -which differ on;y in the size of the ring at cyclo-C6 and cyclo-C7 respectively -had widely differing excretion at 75% and 53% respectively. Faecal excretion of the other 4 compounds ranged from 46% to 48% over the same time scale. The small difference in faecal excretion of JM269 at the 24 and 96 hour time points (41% to 53%)

TABLE 6.3

Plasma concentration/time data (AUC)

COMPOUND	R	R_1	AUC μgPt/ml/hr		ELIMINATION HALF-LIVES PLASMA		
	Peak Plasma concentration μPt/ml		TOTAL Pt 0-96hr	FREE Pt 0-7hr	TOTAL (hr) ß	α	FREE ß(hr)
JM216	4.9	Cyclo-C6	CH_3	108	0.7	21.7	167	>7
JM221	4.4	Cyclo-C6	C_3H_7	120	3.2	18.8	20	>7
JM269	1.9	Cyclo-C7	CH_3	59	0.3	31.0	172	>7
JM272	3.7	Cyclo-C6	$CH(CH_3)_2$	83	4	21.3	36	>7
JM273	15.1	Cyclo-C6	$(CH_3)_3$	105.7	1.43	17.3	35	>7
JM274	1.94	Cyclo-C6	C_4H_9	48	0.8	44.6	35	20.6

- n = 3-6 mice per time point
  reversibly bound fraction was <10% total platinum
- Area under the concentration v.time curve (AUC) measured as μPt/ml/hour

TABLE 6.4

Recovery of compounds in faeces over 96 hours in mice*

COMPOUND	% DOSE DELIVERED 0-24 hr	0-96 hr
JM216	36±3.0	75±13
JM221	13.8±10	48±13
JM269	41±9.4	53±8
JM272	14.5±3.2	47±10
JM273	23.3±18	46±18
JM274	33±5.2	46±8

* 3 mice treated with each drug

suggests that it does not undergo extensive biliary recirculation. Conversely, the high faecal excretion of JM216 (36% at 24 hours and 75% at 96 hours) suggests either that it is poorly absorbed when delivered orally (but see total platinum plasma concentrations above), or that it undergoes extensive biliary recirculation. The same process would account for the slow excretion of JM272 (R = cyclo-C6, $R_1 = OCOCH(CH_3)_2$) where the faecal content increased from a low 14.5% at 24 hours to an average value of 47% after 96 hours.

Table 6.5 details the urinary excretion of each of the six compounds. At 24 hours post administration, the % dose excreted ranged from 5.5-18.2%. At the 96 hour time-point, platinum excreted in the urine ranged from 9% (JM274 R = cyclo-C6, $R_1 = OCOC_4H_9$) to 27% (JM221 R = cyclo-C6, $R_1 = OCOCH_3$). The low urinary excretion of JM274 was suggestive of poor absorption of the compound following oral administration. This concurs with the plasma concentration versus time data for JM274 where the AUC for total platinum was lowest of the six compounds at 48μgPt/ml/hr. The high renal excretion of JM221 (27% at 96 hours) indicated that the compound was well absorbed and this was supported by the plasma platinum concentration v. time data (AUC highest at 120μgPt/ml/hr). JM273 (R = cyclo-C6, $R_1 = OCOC(CH_3)_3$) showed the second highest AUC for free platinum (1.43μgPt/ml/hr; t1/2 35.5 minutes) consistant with its good absorption pattern - peak plasma concentration 15.1μgPt/ml and urinary excretion 20% at 96 hours.

TABLE 6.5

Recovery of compounds in urine over 96 hours in mice*

COMPOUND	% DOSE DELIVERED 0-24 hr	0-96 hr
JM216	11.3±4.1	18±5.9
JM221	18.2±7.0	27±6.8
JM269	6.7±0.5	12±1.91
JM272	15.5±4.6	25±4.1
JM273	13.3±6.6	20±7.9
JM274	5.5±3.8	9.0±3.7

* 3 mice treated with each drug

Table 6.6 is a summary showing total faecal and urinary excretion of each compound over the 96 hour study period. JM216 was the compound most completely recovered - 93% after oral dosing. This is despite the plasma concentration data which demonstrated the half life of JM216 to be intermediate at 21.7 hours (total Pt). The range of total recoveries of the other five compounds at 96 hours - from 55% to 75% -suggests that they are extensively protein bound.

In Table 6.7, the excretion and absorption data for each of the six compounds is compared with the therapeutic index as previously calculated from results obtained in the ADJ/PC6 xenografts. The % excreted in the faeces is calculated for the 48 hour time point. The % absorbed is calculated as the amount not excreted in the faeces ie: % faecal excretion subtracted from a total of 100%. The high therapeutic index (311) for JM274 ($R = $cyclo-C6, $R_1 = OCOC_4H_9$) is associated with a relatively low absorption at 55%. The plasma concentration versus time data (AUC) are listed for the 48 hour time point in this table to relate them to the faecal excretion over the same time period.

JM269 and JM273 are more interesting compounds when these data are analysed since they are both well absorbed ($>70\%$) and demonstrate good antitumour activity with low toxicity (TI's 269 and 181 respectively).

Table 6.8 is included for easy reference to the antitumour activity of

TABLE 6.6

Total recovery of each platinum compound (96 hours)

COMPOUND	STRUCTURE	% DOSE DELIVERED (URINE + FAECES)
JM216	R = cyclo C6 $R_1 = CH_3$	93
JM221	R = cyclo C6 $R_1 = C_3H_7$	75
JM269	R = cyclo C7 $R_1 = CH_3$	65
JM272	R = cyclo C6 $R_1 = CH(CH_3)_2$	72
JM273	R = cyclo C6 $R_1 = (CH_3)_3$	66
JM274	R = cyclo C6 $R_1 = C_4H_9$	55

TABLE 6.7

Comparison between excretion/absorption and anti-tumour activity for the 6 lead compounds

COMPOUND	% excretion faeces (0-48 hrs)	Total Pt (0-48hrs)	Free Pt (0-7 hrs)	% Absorption (100-faeces)	TI
JM216	59	78	0.7	41	57
JM221	30	168	3.2	70	54
JM269	48	12	0.3	52	269
JM272	31	70	4	69	59
JM273	30	72	1.43	70	181
JM274	45	16	0.8	55	311

TABLE 6.8

Antitumour activity of the dicarboxylate dichloride PtIV mixed amines against ADJ/PC6 tumour xenografts.

oral administration

COMPOUND NO	LD50	ED90	TI
216	330	5.8	57
221	280	5/2	54
269	2690	10.4	269
272	170	2.9	59
273	670	3.7	181
274	1120	3.6	311
Cisplatin	140	24	6

the six compounds following oral delivery to mice bearing the ADJ/PC6 tumour, as discussed in Chapter 5.

Figure 6.8 details in histogram form the change in haemoglobin for each compound delivered at LD10, 0.5LD10 and 0.25LD10 doses. Results are expressed as a percentage of the haemoglobin levels in control mice. None of the six compounds induced a fall below 76% of the pretreatment level at the nadir point (Day 7).

Figure 6.9 demonstrates the platelet counts for the compounds at the nadir (Day 10 for all compounds except JM272 - Day 7). Again counts are expressed as a percentage of those measured in the control group of mice. Toxicity is expressed according to WHO criteria (WHO 1979). At the LD10 dose, there was significant (WHO Grade IV) thrombocytopenia after JM221, JM269, JM273 and JM274. JM216 and JM272 induced Grade III thrombocytopenia. At the 0.5LD10 dose, Grade II toxicity was seen for all six compounds, and there was little difference between these levels and those following the 0.25LD10 dose.

The total white cell count (WCC) at the nadir following delivery of each compound is shown in Figure 6.10.For all except one of the compounds, the lowest counts occurred on Day 4. The nadir count for JM274 was seen on Day 7 after dosing. There was an 80% reduction in WCC, when compared with control mice, after dosing with five compounds at the LD10 dose. The exception was JM274 where the result was 43% +/_22. The standard

Figure 6.8 Haemoglobin Count as % of Control
Day 7

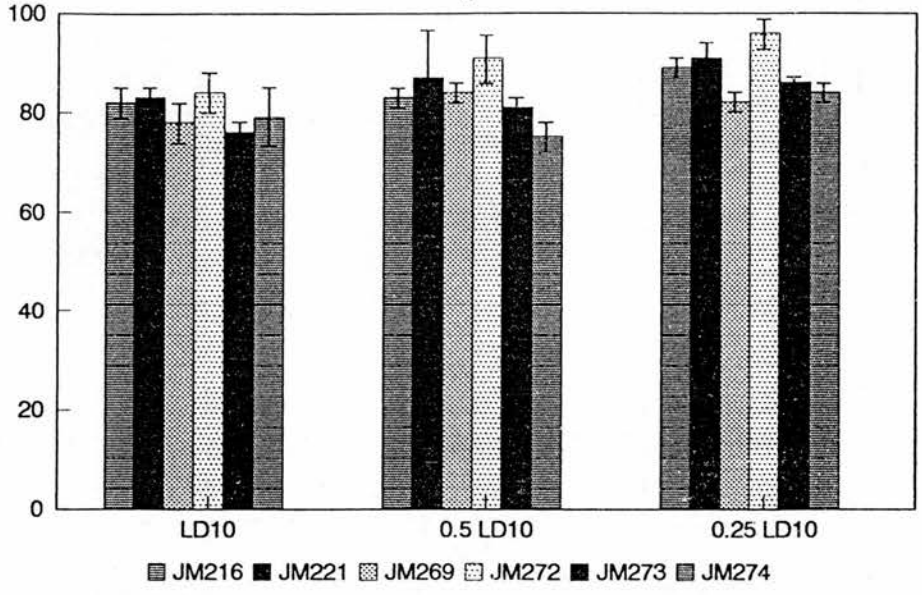
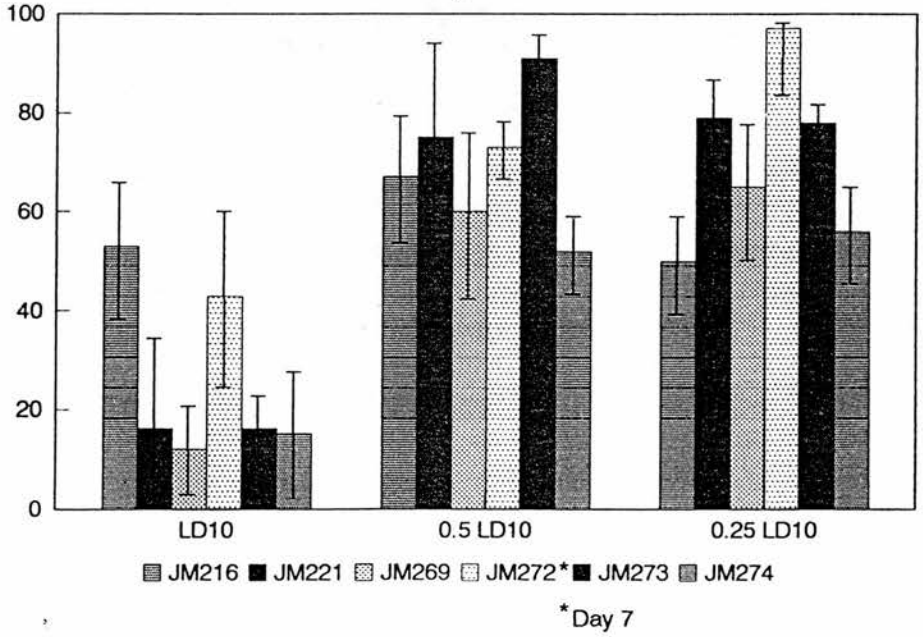


Figure 6.9 Platelet Count as % of Control
Day 10



*Day 7

Figure 6.10 White Cell Count as % of Control
Day 4

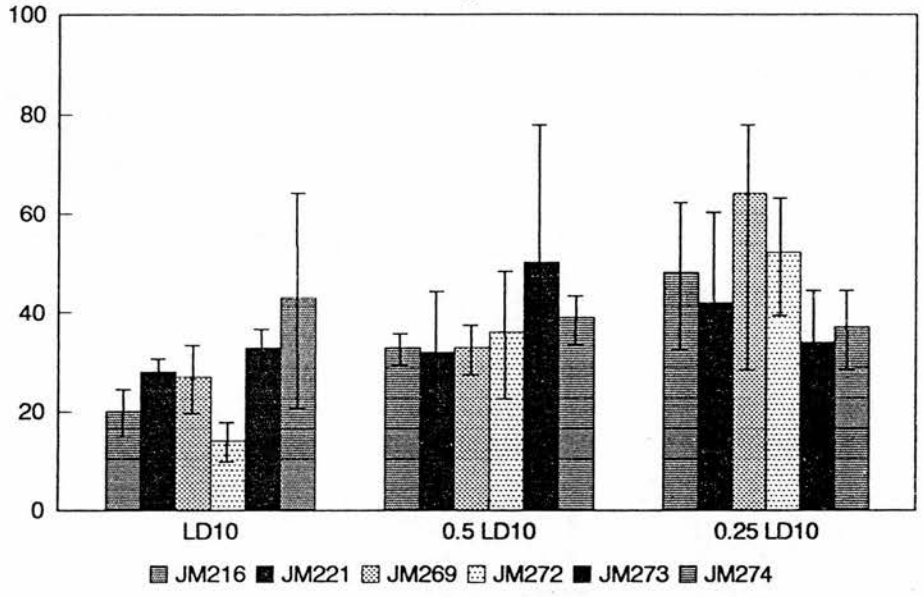
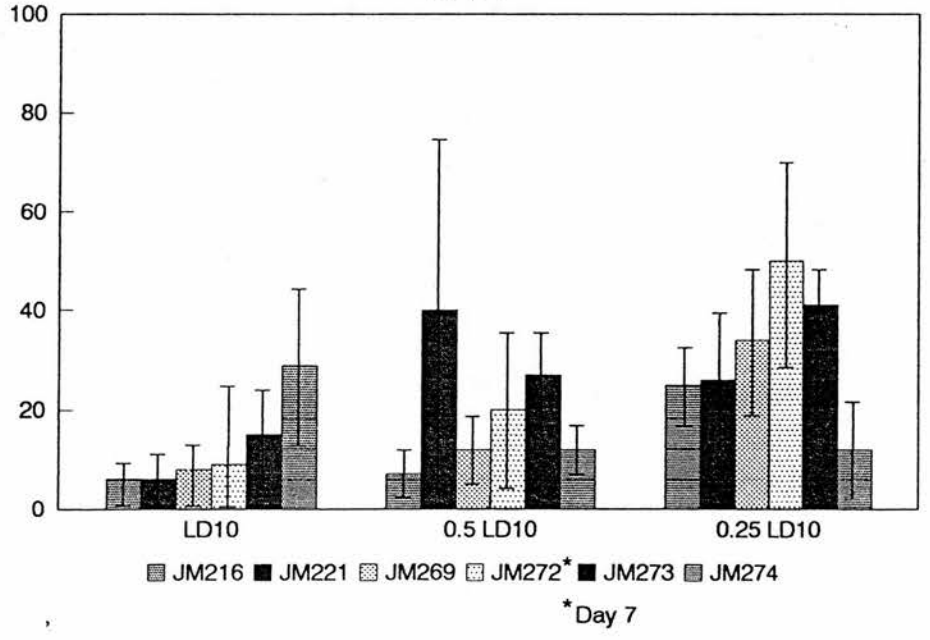


Figure 6.11 Neutrophil Count as % of Control
Day 4



deviation on this result is larger than for the other five compounds. Leucopenia was also moderately severe (from 34 - 64% of control values) following the 0.25LD10 doses, and it is noteworthy that JM273 and JM274 were equally myelosuppressive at LD10 and 0.25LD10 doses.

Figure 6.11 is a histogram of the neutrophil count for each platinum IV dicarboxylate on Day 4 when differential counts were at the nadir (Day 7 for JM274). In all cases there was severe neutropenia at the LD10 dose. JM221 appeared to induce less neutrophil suppression following 0.5LD10 but the error bars in the analysis preclude accurate comment.

Figures 6.12 and 6.13 detail the temporal profile of platelet counts for each lead compound. Following the LD10 dose, recovery of the platelet count to >70% of the control values was seen on Day 15 for JM216, JM221 and JM272. Recovery was delayed in JM273 (80% of pretreatment levels on Day 29), and JM274 (70% of pretreatment levels on Day 29). Platelet recovery following dosing with JM269 was very rapid from a low nadir(around 10% of pretreatment levels) as late as Day 15. For the majority of compounds the thrombocytopenia was less severe and of much shorter duration following the 0.5LD10 and 0.25LD10 doses.

Figures 6.14 and 6.15 show recovery of total WCC with time. The nadir white counts were around Day 10 post treatment with each compound for all doses tested. There was surprisingly little variation in the degree of white cell toxicity between LD10 and 0.25 LD10 doses. For all drugs except

Figure 6.12 Temporal Variation for Platelets

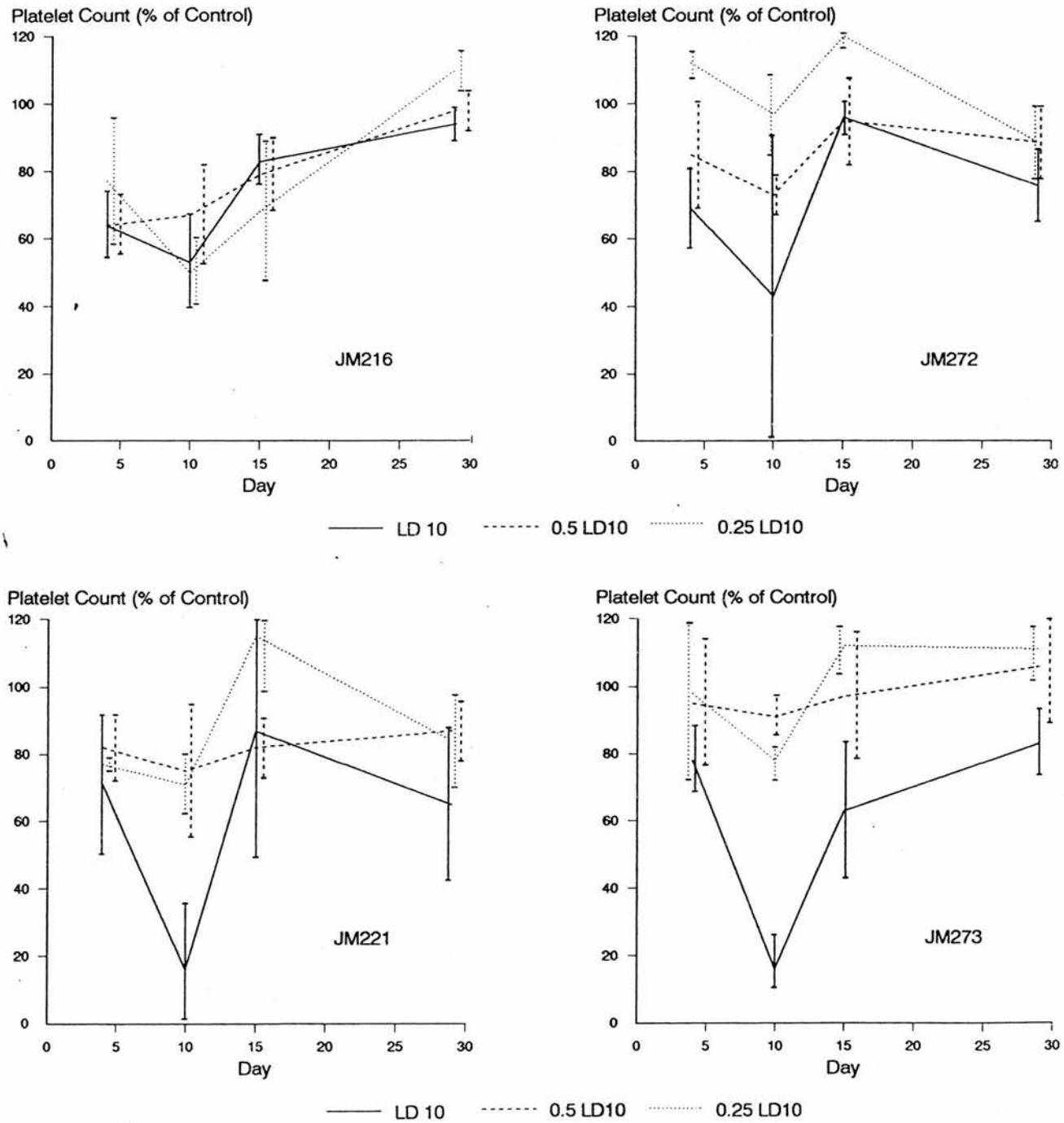


Figure 6.13 Temporal Variation for Platelets

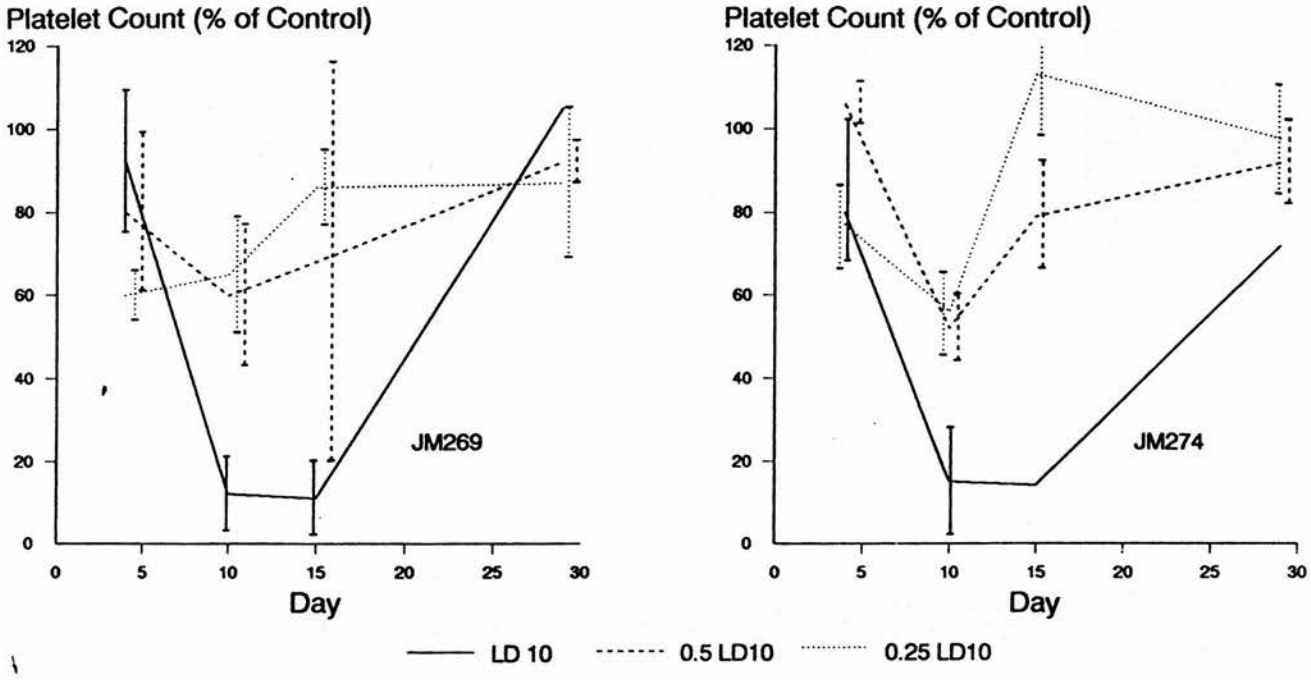


Figure 6.14 Temporal Variation for White Cells

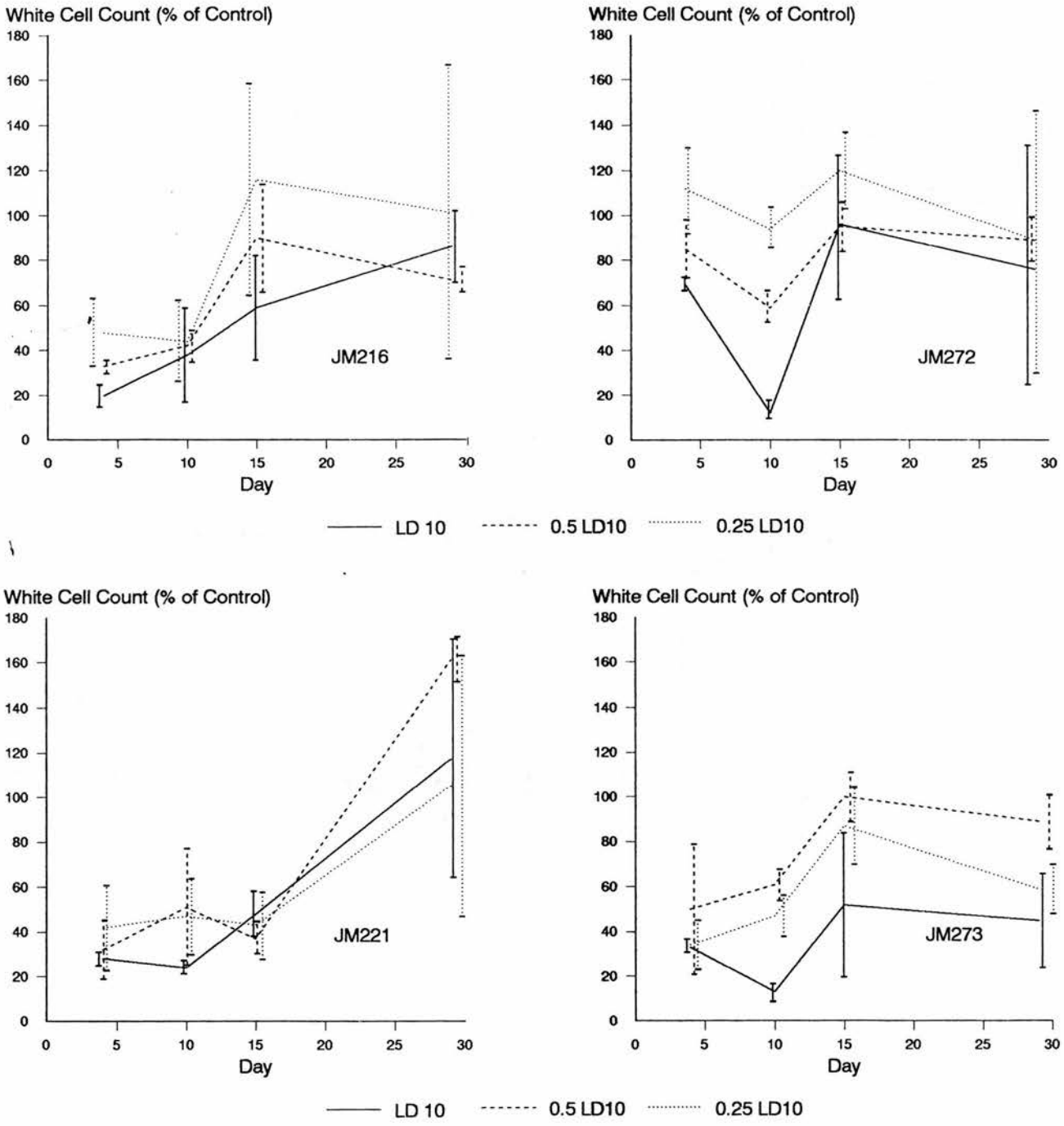
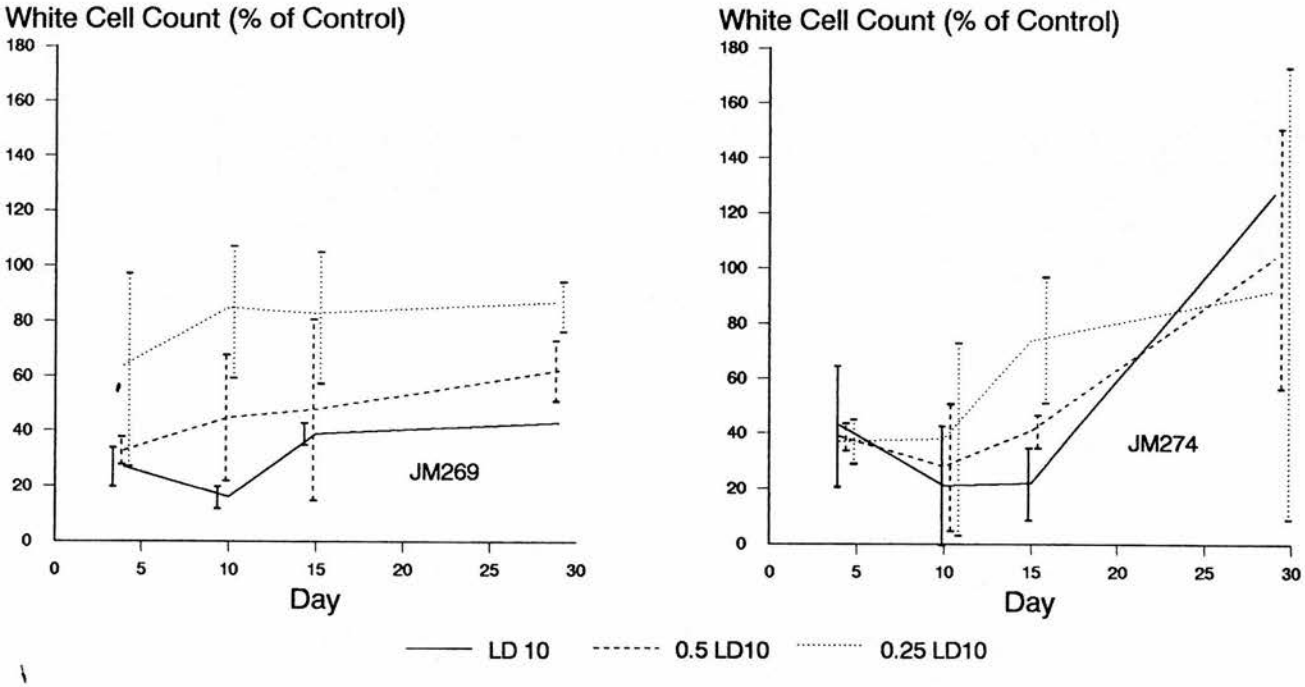


Figure 6.15 Temporal Variation for White Cells



JM269 and JM273, the white cell count had recovered to 70% of more than control values by Day 30. The error bars were wide in most of these experiments, and this explains the finding of the 0.5 LD10 dose being apparently less toxic than the 0.25 LD10 dose for JM273. Recovery of white count was most rapid for JM216 and JM273 although there was an apparent later dip between Days 15 and 30 with JM273.

6.4 Discussion.

It has been reported that the relatively unstable chemical structure of cisplatin mitigated against it being useful as an orally administered drug (Siddik et al, 1984), and indeed the bioavailability of a given dose was only 25% when administered orally [Harrap et al, 1981]. The more stable structure of carboplatin might have made it a more suitable candidate for oral delivery, but studies refuted this [Hennik et al, 1989]. The bioavailability of carboplatin after an oral dose was estimated to be about 15% in mice but $<10\%$ in man. The rapid gastrointestinal (GI) transit time in the mouse of orally administered cisplatin and carboplatin may have contributed to the poor bioavailability (Morgan, SE PhD thesis 1989). It might be expected that the high therapeutic indices in the ADJ/PC6 tumour-bearing mice seen following oral administration of the six lead compounds would correlate with an improvement in absorption and bioavailability. The increased lipophilicity of the compounds would also be likely to increase their absorption following oral delivery.

It had previously been estimated that the oral bioavailability of several of the ammine/amine platinum IV dicarboxylates ($PtCl_2(OCOR_1)2NH_3RNH_2$ where R_1 is aliphatic and R is alicyclic) could be as high as 90% following oral administration [Harrap et al, 1991]. When comparing the data for cisplatin and carboplatin with those for the dicarboxylates, it was evident that for the dicarboxylates the ED90's were similar following either intraperitoneal or oral dosing, yet toxicity (LD50) was decreased 10-fold.

The LD10 dose in mice following oral delivery of the comounds varied greatly for the six alicyclic ammine compounds tested, from 100mg/kg for JM272 (where $R_1 = OCOCH_2(CH_3)_2$, R = cyclo-C6) to 1.5g/kg for JM269 where $R_1 = OCOCH_3$ and R = cyclo-C7 (Table 6.1). The TI's in PC6 tumour-bearing mice for these compounds after oral administration (Table 6.7) were 59 and 269 respectively, reflecting the comparatively low toxicity of JM269 compared with JM272. Structural comparisons revealed that the two cyclohexylamine compounds JM216 ($R_1 = OCOCH_3$) and JM272 ($R_1 = OCOCH_2(CH_3)_2$) differ only in their axial ligand (Figure 6.1). The increase in axial chain length conferred an increase in the LD50 and a reduction in the ED90 for JM272 compared with JM216 whereas the TI's for these two drugs were similar (59 and 57).

The relative absorptions of JM216 and JM272 were also compared in an attempt to explain the differences resulting from the structural changes. From the urinary/faecal data it was seen that a larger proportion of the administered dose of JM216 was recovered in the faeces over 24 hours (36%) than after JM272 (14.5%) (Table 6.3). As previously stated, a high faecal recovery for a compound suggests that it is relatively poorly absorbed. Considering the urinary excretion of the two compounds, 21% of an oral dose of JM272 was excreted in 48 hours compared with 16% of the JM216 (Table 6.4). The total platinum AUC for JM216 was slightly larger than that for JM272 (108 μgPt/ml/hour and 83 μgPt/ml/hour respectively) (Table 6.2). Only when the AUC values for the free platinum were compared was a large difference found, with JM216 reaching 0.7

μgPt/ml/hour and JM272 4.0 μgPt/ml/hour and little difference in the half life (Table 6.2). These figures suggest that much more free and potentially active platinum was present in plasma after oral administration of JM272 than following JM216, thus explaining the larger urinary recovery of the former compound. The higher circulating free platinum levels may therefore reflect the increased potency (low ED90) and increased toxicity (lower LD50) of this compound compared with JM216. It therefore appears that a longer axial chain length is associated with better absorption of an oral PtIV dicarboxylate compound.

A reduction in absorption was seen in those compounds with acetato axial ligands as opposed to a congeraxial ligand. This finding was confirmed by the cycloheptyl analogue JM269. JM269 was less potent than JM272 (ED90 10.4 v 2.9), with a greater than 15-fold increase in LD50 resulting in a high TI (268 v. 59)(Table 6.7). The pharmacokinetic data for JM269 revealed that only 6.7% was excreted in the urine over the first 24 hours post administration (Table 6.4), with 41% in the faeces for the same period (Table 6.3). The AUC of JM269 for total platinum was only 12 μgPt/ml/hour and for free Pt 0.3 μgPt/ml/hour at 48 hours with the amount absorbed second lowest of all of the six compounds at 52% (Table 6.6). As stated above, the structural difference between JM269 and JM216 is only in the R group - cyclo-C7 for JM269 and cyclo-C6 for JM216. Both compounds have a methyl (CH_3) group at R_1. The TI from the ADJ/PC6 experiments revealed a 5-fold difference between the compounds (269 for JM269, 57 for JM216) due mainly to the very low toxicity of JM269 (LD50

2690) (Table 6.7). The LD10 data also varied greatly (Table 6.1), calculated at 200mg/kg for JM216 and 1500mg/kg for JM269. The pharmacokinetic data revealed better absorption of the cyclo-C7 compound JM269 with less of the administered dose recovered in the faeces at 96 hours (53% JM269 versus 75% JM216) (Table 6.3) yet higher urinary excretion of JM216 (18% versus 12% for JM269) (Table 6.4). JM216 underwent extensive enterohepatic circulation as shown by the difference in faecal excretion between 24 and 96 hours (36% and 75%) (Table 6.4). The total amount of platinum recovered was 93% (JM216) and 65% (JM269), suggesting that there was extensive protein binding of the latter compound (Table 6.5). The peak plasma concentrations also suggested better absorption of JM269 (4.9μgPt/ml versus 1.9μgPt/ml) (Table 6.2). However the plasma drug concentration/time curve revealed a better AUC for JM216 over 96 hours (108μgPt/ml/hour versus 59μgPt/ml/hour for JM269) (Table 6.2). There was a less significant difference between the free platinum AUC - 0.7μgPt/ml/hour for JM216 versus 0.3μgPt/ml/hour for JM269 (Table 6.2). There was also a small difference in the elimination half lives for the total platinum - 21.7 hours (JM216) versus 31 hours (JM269) (Table 6.2).

The conclusion from these experiments was that although less of the JM216 was absorbed, it was less protein bound and there was an increased amount of the free (active) platinum around following the oral dose, mitigating in favour of the cyclohexyl ring structure.

Oral administration of JM274 ($R_1 = OCO(CH_2)_3CH_3$, R = cyclo-C6) to ADJ/PC6 tumour-bearing mice resulted in a good TI of 311 (LD50 1120 and

ED90 3.6) consistent with good potency as an antitumour agent and low toxicity (Table 6.7). The urinary recovery was the poorest of all the investigated compounds at only 5.5% in the first 24 hours post oral dosing (Table 6.4). A moderate 33% of the dose was recovered in the faeces at 24 hours (Table 6.3). Despite the encouraging LD50 and ED90, the total platinum AUC (48 μgPt/ml/hr) and free platinum AUC (0.8 μgPt/ml/hr) were among the lowest for all of the compounds tested, implying with the low urinary excretion that the compound was poorly absorbed (Table 6.2). Evaluation of the sister compound JM273 was carried out. Here, although both compounds have a cyclo-cyclo-C6 group on the amine ligand, the axial ligand of JM273 is a tertiary butarato ($R_1 = OCOC(CH_3)_3$) as opposed to a straight chain butyrato ligand ($R_1 = OCOC_4H_9$) for JM274. The TI of JM273 (181) was the second best of the six compounds (Table 6.7). Urinary excretion was 11.4% of the oral dose in the first 24 hours (Table 6.4), and the peak blood level was 15.1 μgPt/ml compared with 1.94 μgPt/ml for JM274 (Table 6.2). The AUC for total platinum from 0-48 hours was 72 μgPt/ml/hour following JM273, a marked improvement on the result of 16μgPt/ml/hr for JM274 (Table 6.6). JM273 demonstrated an AUC (0-7 hours) for free platinum of 1.43 μgPt/ml/hour and this was also significantly better than the 0.8 μgPt/ml/hour seen with JM274 (Table 6.6).

It was shown (Table 6.5) that over 96 hours the total urine and faecal recovery of JM273 was 66% compared with 55% for JM274. The elimination half life of total platinum was 17.3 hours (JM273) compared with 44.6 hours for the sister compound JM274 (Table 6.2).

These values were the shortest and longest half lives recorded for the six

lead compounds and suggest that the straight chain of the R_1 group on JM274 results in poorer absorption and more extensive protein binding of the compound.

JM221 (R = cyclo-C6, $R_1 = OCOC_3H_7$) differs from JM274 only in the one extra carbon on the latter's aliphatic chain. There was a large difference in the TI (57 versus 311) (Table 6.1) and a notable difference in LD10 in non-tumour bearing mice (150mg/kg vesus 400mg/kg) (Table 6.1), yet rather less variation in the % of dose absorbed (41% versus 55%) (Table 6.6). The most impressive change with lengthening the aliphatic chain was in the plasma concentration versus time curve, since the AUC for total platinum at 96 hours was the highest for all six compounds at 120μgPt/ml/hour for JM216 and lowest for all compounds at 48μgPt/ml/hour for JM274 (Table 6.2). There was a corresponding difference in the free platinum (3.2μgPt/ml/hour JM216 versus 0.8μg/ml/hour JM274).

The conclusion from these data is that the absorption peaks with an aliphatic chain of three carbons, but it should be noted that the elimination half life of the longer chain was prolonged, compatable with increased protein binding of the long chain compound.

The biphasic elimination half life for the six PtIV dicarboxylates was expected from the data for the parent platinum compounds cisplatin and carboplatin [Siddik et al, 1988], and there was a relatively narrow range of values for all six compounds. The fact that the terminal elimination half lives for total platinum approach two days (Table 6.2) suggests that all of

the compounds are extensively protein bound. Elimination of free platinum is biphasic with a rapid alpha half life of between 20 and 172 minutes and a long beta half life of greater than 7 hours. These findings are of importance when it comes to repeat dosing with the compounds, since one may predict cumulative toxicity when the terminal elimination is greatly prolonged.

The half life of free carboplatin is in the order of 10 to 87 minutes, and that of cisplatin is around 10 minutes following iv administration. The orally delivered PtIV dicarboxylates are therfore around in the circulation for a considerablly longer period of time than are the parent platinum II drugs which are currently in use. Equally, the decay of the free platinum is monophasic for cisplatin and carboplatin and no platinum was detectable after 24 hours.

Measurement of the haematological toxicity confirmed that the PtIV dicarboxylates were all toxic to the bone marrow. Thrombocytopenia was most significant at LD10 doses of JM221, 269, 273 and 274 (Figure 6.9). The platelet nadir (around 20% of control values) was on Day 10 for all compounds except JM272 where it plateaued on Day 7. JM216 induced least severe thrombocytopenia, the nadir reaching just under 60% of the control. Recovery to at least 60% of control values was seen following the LD10 dose in every case by day 30 (Figures 6.12 and 6.13).

Leucopenia was also most evident at the LD10 doses of the drugs (neutrophils $<20\%$ of control values for all compounds excepting JM274), but total neutrophil counts also fell to less than 40% of controls following the 0.5LD10 doses. For all drugs except JM274, the white cell nadir was on Day 4. The nadir post JM274 was on day 7. Neutrophils were severely suppressed even at 0.25LD10 doses (15 to 50% of controls). JM274 was the most markedly toxic to neutrophils at this dose level but as with the other compounds the effects were relatively short-lived with recovery by Day 15 in all cases Figures 6.14 and 6.15).

Anaemia was not a significant toxicity with any of the drugs, all values remaining at or above 80% of the control values on day 7 (Figure 6.8).

In summary, severe neutropenia on day 4 at LD10 and 0.5LD10 doses and moderate to severe thrombocytopaenia on day 10 at LD10 doses were seen after treatment with all drugs. Recovery of the counts after a single adminisration was complete in every case by Day 28, but it could be postulated that haematological toxicity may be dose-limiting for the six lead novel platinum compounds.

Further analysis of the PtIV dicarboxylate compounds proceeded with an analysis of their emetogenic potential in the ferret and assessment of nephro- and hepatotoxicity. The data resulted in elimination of several of the six lead compounds and selection of JM216 as the preparation most suitable for evaluation in Phase I clinical studies.

CHAPTER 7

CONCLUSIONS.

The aims of this thesis were to investigate the mechanisms of resistance to platinum based chemotherapy and to select a novel agent for administration by the oral route.

Using the L1210 murine leukaemia and its newly developed platinum resistant variants as *in vitro* tumour models, it was established that one of the means by which the cells become resistant is through a reduction in the uptake of platinum. There was no evidence of an increase in efflux of platinum from the cells and this mitigated against the p170 membrane glycoprotein playing a role in these cells' resistance. Colony assay experiments demonstrated that resistant L1210 cells were better able to tolerate increased concentrations of intracellular platinum than were the parent sensitive cells and it was established in later experiments that there was indeed less platinated DNA in resistant cells when compared with sensitive L1210 exposed to equivalent doses of platinum. Glutathione (GSH) concentrations were measured in both the parent line and in the resistant L1210. There was a slight increase in the total GSH concentration in the L1210/carbo cell line when compared with the sensitive, L1210/cis and L1210/tetra lines, but no significant difference in GSH levels was found after exposing the cell lines to the IC50 concentrations of cisplatin, carboplatin or tetraplatin. Depletion of intracellular GSH through addition of buthionine sulphoximine (BSO) to the cell cultures did not confer a return of

sensitivity to the platinum resistant cells, confirming that GSH does not play a role in platinum resistance in L1210 cells *in vitro*.

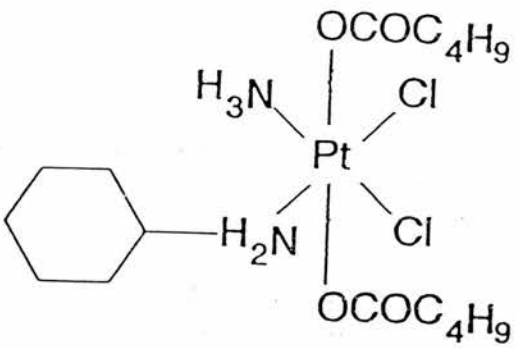
In order to identify a novel platinum complex suitable for oral administration, six compounds were tested for antitumour activity against the L1210 parent and resistant cell lines, and against human ovarian carcinoma cells in vitro. The ammine/amine platinum IV dicarboxylate compounds were selected on the basis of their potency in inhibiting growth of the ADJ/PC6 tumour *in vivo*. The six lead compounds all exhibited antitumour activity against the platinum resistant L1210 lines (L1210/cis, L1210/carbo and L1210/tetra). Studies of the drugs' effectiveness against six human ovarian carcinoma cell lines *in vitro* revealed that intrinsically cisplatin resistant cell lines were relatively more sensitive to the mixed amine platinum IV dicarboxylates. Optimal antitumour activity was related to the presence of a cyclohexyl alicyclic ring on the amine ligand with an axial chain length of 6 or 7 carbons.

The absorption of each compound was established following its oral administration to non tumour-bearing female Balb-C mice. The plasma concentration of drug was measured at time points up to 96 hours, with rate and percentage excretion via the faecal and oral routes also measured to establish a full pharmacokinetic profile for all six drugs. The compound JM274 (Figure 7.1) demonstrated a high therapeutic index (311) following oral delivery to mice bearing the ADJ/PC6 tumour, but it was demonstrated to have the lowest free (potentially active) platinum in plasma and a high

percentage of recovery in the faeces, indicative of poor absorption. Since the toxicity of JM274 was low in the ADJ/PC6 experiments (LD50 = 1120mg/kg), these initial studies suggested that it may be a favourable candidate for clinical evaluation. Haematological toxicity testing showed that neutropenia and thrombocytopenia were evident at days 4 and 10 respectively following treatment with LD10 and 0.5LD10 doses of all compounds.

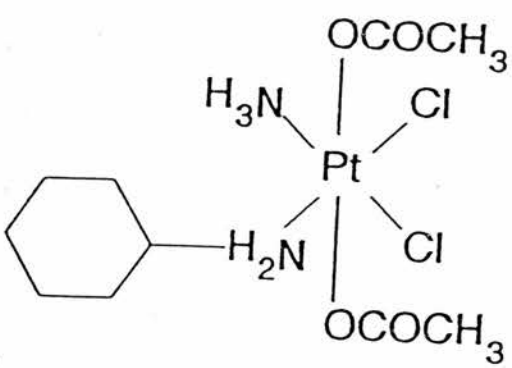
Although JM274 looked promising in these preliminary screening studies, experiments of emetogenicity in the ferret model revealed that JM274 induced unacceptable amounts of vomiting. It was therefore necessary to compromise on efficacy when selecting the agent for further evaluation in the clinical setting, and JM216 (structure Figure 7.2) was the candidate selected to undergo testing in Phase 1 clinical trials which are currently in progress.

FIGURE 7.1



JM274

FIGURE 7.2



JM216

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